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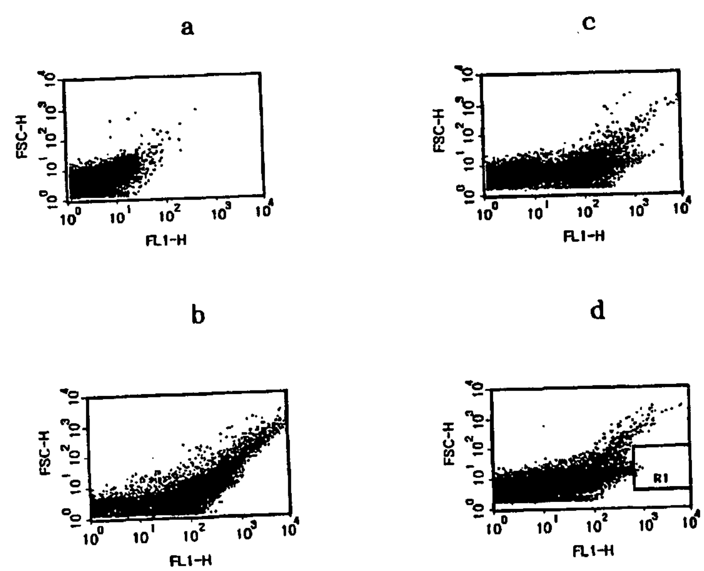
(54) Title: METHOD FOR EXPRESSION OF PROTEINS ON SPORE SURFACE



(57) Abstract: The present invention relates to a method for display of proteinson spore surface and a method for improving protein with rapidity using the same, which comprises the steps of (i) preparing a vector for spore surface display comprising a gene construct containing a gene encoding spore coat protein and a gene encoding a protein of interest, wherein, when expressed, the gene construct expresses a fusion protein between the spore coat protein and the protein of interest, (ii) transforming a host cell with the vector for spore surface display; (iii) displaying the protein of interest on a surface of a spore of the host cell; and (iv) recovering the spore displaying on its surface the protein of interest.

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METHOD FOR EXPRESSION OF PROTEINS ON SPORE SURFACE

FIELD OF THE INVENTION

The present invention relates to a method for display of proteins on spore surface, in particular to a method for surface display using spore coat proteins as surface display motif and a high throughput method for improving protein.

DESCRIPTION OF THE RELATED ART

The technology of surface display in which organism displays on its surface the desired proteinaceous substance such as peptide and polypeptide has wider application fields depending on the types of protein displayed or host organism (Georgiou *et al.*, 1993, 1997; Fischetti *et al.*, 1993; and Schreuder *et al.*, 1996). The gene of protein to be displayed is contained in host organism and thus the host can be selectively screened using the characteristics of the protein displayed, thereby obtaining the desired gene from the selected host with easiness. Therefore, such surface display technology can guarantee a powerful tool on molecular evolution of protein (see WO 9849286; and U.S. Pat. No. 5,837,500).

High-Throughput Screening

For instance, phage displaying on its surface antibody

having desired binding affinity is bound to immobilized antigen and then eluted, followed by propagating the eluted phage, thereby yielding the gene coding for target antibody from phage (U.S. Pat. No. 5,837,500). The bio panning method described above can provide a tool to select target antibody by surface displaying antibody library on phage surface in large amount and comprises the consecutive steps as follows: (1) constructing library; (2) surface displaying the library; (3) binding to immobilized antigen; (4) eluting the bound phage; finally (5) propagating selected clones.

The technology of phage surface display has been found to be useful in obtaining the desired monoclonal variant form enormous library (e.g., 10^6-10^9 variants) and thus applied to the field of high-throughput screening of antibody. Antibody has been used in various fields such as therapy, diagnosis, analysis, etc. and thus its demand has been largely increased. In this context, there has been a need for novel antibody to have binding affinity to new substance or catalyze biochemical reaction. The hybridoma technology to produce monoclonal antibody has been conventionally used so as to satisfy the need. However, the conventional method needs high expenditure and long time for performance whereas the yield of antibody is very low. In addition to this, to screen novel antibody, more than 10^{10} antibody libraries is generally used, as a result, the hybridoma technology has

been thought to be inadequate in finding antibody exhibiting new binding property.

Many researches has focused on novel methods which is easier and more effective that the bio panning method described above and then developed novel technologies performed in such a manner that libraries are displayed on surface of bacteria or yeast and then cells displaying target protein is sorted with flow cytometry in a high-throughput manner. According to the technology, antigen labeled with fluorescent dye is bound to surface-displaying cell and the antibody having the desired binding affinity is isolated with flow cytometry capable of analyzing more than 10^8 cells a hour. Francisco, et al., have demonstrated the usefulness of microbial display technology by revealing that surface-displayed monoclonal antibody could be concentrated with flow cytometry at rate of more than 10^5, finally more than 79% have been proved to be the desired cells (Daugherty *et al.*, 1998).

Live Vaccine

The surface display technology mentioned above can display antigen or fragment thereof and hence provide a delivery system for recombinant live vaccine. Up to now, attenuated pathogens or viruses have been predominantly employed as vaccine. Particularly, the bacteria have been

found to express antigen intracellularly or extracellularly or on its cell membrane, thereby delivering antigen to host cell. The surface-displayed live vaccine induces a potential immune reaction and expresses continuously antigen during propagation in host cell; therefore, it has been highlighted as novel delivery system for vaccine. In particular, pathogen-derived antigenic epitope displayed on surface of nonpathogenic *E. coli* or Salmonella is administered orally in viable form and then exhibits to induce immune reaction in more continuous and powerful manner (Georgiou *et al.*, 1997; and Lee *et al.*, 2000).

Whole Cell Bioconversion

Whole cell as biocatalyst displaying on its surface enzyme capable of catalyzing chemical reaction can avoid necessities for direct expression, isolation and stabilization of enzyme. In case of expressing enzyme in cell for bioconversion, the cell is compelled to recovery and chemical (e.g., toluene) treatment to ensure impermeability of substrate. In addition, the lasting use renders the enzyme inactive or gives a problem on transference of substrate and product, thus dropping the productivity of overall process.

The above-mentioned shortcomings can be removed using enzyme displayed on cell surface (Jung et al, 1998a: 1998b).

With whole cell displaying on its surface phosphodiesterase, organophosphorous-typed parathion and paraoxon with higher toxicity can be degraded, which is a typical example representing the applicability of cells displaying enzyme to environmental purification process (Richins et al., 1997).

Antipeptide Antibody

Martineau et al. have reported a highly simple method for production of antipeptide antibody using surface display technology of *E. coli* (Martineau *et al.*, 1991). As described, the desired peptide is displayed on the protruding region of MalE and outer membrane protein, LamB and then whole cell or fragmented cell is administered to animal so as to generate antipeptide antibody. The method makes it possible to produce antibody with avoiding chemical synthesis of peptide and its linkage to carrier protein.

Whole Cell Absorber

To immobilize antibody or polypeptide on suitable carrier, which is useful in absorption chromatography, several subsequent steps must be performed, for example, protein production by fermentation, isolation of protein in pure form, and immobilization on a carrier. Generally, it is difficult to prepare the bioabsorber.

As absorber, a whole cell displaying absorption protein

has been developed. The whole cell absorber known mostly is *Staphylococcus aureus* displaying on its surface protein A naturally, which has a high binding affinity to Fc domain of mammalian antibody. Currently, novel method has been proposed to remove and recover heavy metals, which employs metallothionein or metal-absorption protein displayed on microbial cell surface in large amount (Sousa et al., 1996, 1998; and Samuelson et al., 2000). The method is more effective in removing and recovering heavy metals from contamination source in comparison with the conventional method using metal-absorption microbes.

As understood based on the matters described above, in order to display foreign protein on cell surface, a suitable surface protein and foreign protein must be linked each other in gene level to express fusion protein, and the fusion protein should pass stably across inner membrane of cell to be attached to cell surface. Preferably, the surface protein having the following characteristics is recommended as surface display motif: 1) existence of secretory signal enabling passage across inner membrane of cell, 2) existence of target signal enabling stable attachment to cell surface, 3) high expression level on cell surface, and 4) stable expression regardless of protein size (Georgiou et al., 1993).

Therefore, the surface display motif or novel recombinant protein, which meets the requirements described above, should be selected or prepared to develop novel surface display system overcoming disadvantages of the known systems. In addition, the selection of a suitable host cell to display is very pivotal.

Up to date, the developed surface display systems are as follows: phage surface display system (Chiswell and McCarferty, 1992), bacterial surface display system (Georgiou et al., 1993; Little et al., 1993; and Georgiou et al., 1997), surface display system of Gram negative bacteria (Francisco et al., 1992; Fuchs et al., 1991; Klauser et al., 1990, 1992; and Hedegaard et al., 1989), surface display system of Gram positive bacteria (Samuelson et al., 1995; Palva et al., 1994; and Sleytr and Sara, 1997), and surface display system of yeast (Ferguson, 1988; and Schreuder et al., 1996).

In the developed phage display system, the concentration of the desired clone from phage library has been found to be difficult and the antibody selected from phage library displaying has usually exhibited very low expression rate. According to a surface display system of Gram negative bacteria, the incorporation of foreign polypeptide into surface structure results in not only its steric limitation which makes it impossible to have stable membrane protein

(Charbit et al., 1987; and Agterberg et al., 1990) but also drop of the stability of cell outer membrane and its viability. In addition, in surface display system of yeast, because the vector used has usually shown a low rate of transformation, which is unfavorable to surface display of library.

The surface display systems developed have been cooperatively used each other. For example, to screen antibody variant with enhanced binding affinity, a primary screening is performed using phage surface display system and additionally, the secondary screening is carried out using cell surface display system (Georgiou, 2000). However, the phage display technology is encountered to difficulty in concentration of the desired clones from phage library. The reason is that the antibody displayed on phage surface does not show the elution pattern depending exactly on its binding affinity, which is ascribed to avidity of antibody displayed on phage surface. Therefore, there remains a need of novel methods ensuring screening the desired antibody from antibody library.

E. coli as display host, which has been intensively studied, uses generally cell outer membrane protein as surface display motif. However, the over-expression of cell outer membrane protein fused to foreign protein is likely to

bring about structural instability of cell outer membrane, consequently, diving the viability of host cell (Georgiou et al., 1996). To be from the shortcomings, ice-nucleation protein with no effect on viability has been used as display motif, and has been applied to bioconversion process, surface display of enzyme library and screening enzyme variants (Jung et al., 1998a, 1998b; and Kim et al., 1998, 1999, 2000).

The size of library displayed on surface depends on the transformation efficiency of host cell with vector; thus *E. coli* as host has an advantage in view of the size of library to be displayed. Gram positive bacteria as host are relatively rigid and permit stable display of the desired protein; however, transformation efficiency is exhibited low, which results in smaller size of library than *E. coli*.

The host organisms having been developed are likely to be sensitive to a variety of physiochemical treatments, which makes it impossible to select proteins displayed on surface by virtue of direct physiochemical treatment. For example, in screening a variant of antibody with enhanced binding affinity, abrupt change of pH or adjustment of the concentration of base is generally performed to elute the variant, which are found to decrease the viability of phage or bacteria in medium.

In addition, the host organisms used conventionally have

a complicated and weak structure of cell surface, which drops adaptability to extreme environment such as high temperature and high pressure. To employ *E. coli* displaying on its surface enzyme in bioconversion reaction, the cells must have represent stability in bioconversion system. In this context, the surface of *E. coli* displaying on its surface enzyme is generally subject to immobilization, which does not lead to satisfying results (Freeman et al., 1998).

As described above, the known surface display technologies, based on applying fields, have used bacteriophage, Gram negative or positive bacterium, yeast, cilium or mammalian cell as host organism and surface proteins of each organism as surface display motif. However, in the surface display methods having been developed, the host organism does not have resistance to chemicals and physiochemical change such as pH change, and displaying protein on its surface in excess leads to disadvantages in cell surface, finally reducing the viability of host cell largely (Georgiou et al., 1996).

DETAILED DESCRIPTION OF THIS INVENTION

Under such situation, the present inventors have made intensive studies to be from the shortcoming of conventional display methods, and as a result, we have developed novel display system using a spore as host and a coat protein as

motif of surface display. Surprisingly, the developed display system has been found to have excellent stability to a variety of physiochemical stresses in surrounding environment and have much broader applicability.

Accordingly, it is an object of this invention to provide a method for displaying a protein of interest on spore surface using a system for spore surface display.

It is another object of this invention to provide a method for improving a protein of interest using a system for spore surface display.

It is still another object of this invention to provide a method for bioconversion using a system for spore surface display.

It is further object of this invention to provide a method for preparing protein microarray using a system for spore surface display.

It is still further object of this invention to provide a method producing an antibody to antigen in vertebrates using a system for spore surface display.

It is another object of this invention to provide a method for preparing a whole cell absorber using a system for spore surface display.

It is still another object of this invention to provide a microbial transformant for spore surface display of a protein of interest.

It is further object of this invention to provide a spore for spore surface display of a protein of interest.

It is still further object of this invention to provide a vector for spore surface display.

The principle of the present invention lies in the employment of microbial spore as host for surface display and spore coat protein as surface display motif. The present inventors have been compelled to select a system for spore surface display since the the spore has a following advantages (Driks, 1999): 1) a higher heat stability, 2) a significant stability to radioactivity, 3) a stability to toxins, 4) a higher stability to acid and base, 5) a significant stability to lysozyme, 6) a resistance to dryness, 7) a higher stability to organic solvents, 8) a fusion protein between a surface display motif and a protein of interest is displayed on spore surface immediately after expression without secretion in host cell, 9) no metabolic activity, and 10) shorter time for obtaining spore, e.g. within several hours.

In particular, the spore coat proteins used in this invention circumvent a necessity for passage across cell membrane, so that they do not need secretion signal and target signal which are prerequisites of surface display motif, thereby ensuring a surface display of protein such as

β-galactosidase, in orderly fashion, which is difficult to pass across cell membrane.

U.S. Pat. No. 5,766,914 discloses a method of producing and purifying enzymes using fusion protein between cotC or cotD among spore coat proteins of *Bacillus subtilis* and lacZ as reporter. However, as disclosed, a purification method for demonstrating surface display of protein is not recognized to isolate spores specifically. Furthermore, the activity of enzyme expressed has been very low and the display of enzyme on spore surface has never been demonstrated by means of reliable methods such as biochemical, physical and immunological methods. In addition to this, the inner coat protein, cotD is enclosed by outer coat protein of 70-200 nm thickness, which makes it difficult to be exposed to spore surface. In case of fusion protein expression using outer coat protein, cotC, the activity of enzyme is increased by four-fold in comparison with that of cotD; however, the activity, 0.02 U, is considered negligible, in particular, in consideration of industrial scale. Therefore, the matter disclosed in the document above cannot be considered to use and recognize a system for spore surface display. In other words, the patent document cannot be recognized to describe a system for spore surface display. U.S. Pat. Nos. 5,837,500 and 5,800,821 also indicate cotC and cotD as a preferable surface display motif,

and therefore the patent documents cannot be recognized to describe a system for spore surface display because of the reasons mentioned above.

Furthermore, according to the purification method of spore proposed in U.S. Pat. No. 5,766,914, half of the purified resultant has been observed under microscope to have the complex forms between cells harboring spores and cell-lysis matters bound to spores (see Fig. 1; cells with blackish color and long side are those not forming spore and spores is observed to be white and circular), which has been demonstrated by the present inventors. The facts hereinabove reveals possibility to bring about the false results by measuring of the activity of reporter enzyme or analyzing of reporter enzyme with flow cytometry in vegetative cells rather than on spore surface. In contrast, the renografin gradient centrifugation as demonstrated in Examples below allows for the perfect purification of spores (see Fig. 2), thereby measuring the activity of enzyme displayed on spore surface solely.

Observations on lower enzyme activity in several documents including the patents above are likely to be resulted from the following reasons. First, it is suggested that the expression level of coat protein itself is low. The maximum expression levels of CotC and CotD are 40 and 147 Miller Units, respectively, which is considered to be

largely low, in particular, in consideration of CotE of 6021 Miller Units (Zheng L and Losick R., *J. Mol. Biol.* 212:645-660(1990)). Furthermore, it is notable that the amount of enzyme displayed on spore surface has not been reported. Secondly, it is possible that the protein displayed on spore surface is cleaved by protease in host cell. Such suggestion is made based on the fact that at spore-forming stage of *Bacillus subtilis* a variety of proteases are expressed and reconstitution for spore formation is occurred. The suggestion can be demonstrated in Examples below in which a variant lack protease exhibits a much higher enzyme activity displayed on spore surface (see Fig. 7).

Using gene of GFP (green fluorescence protein) as reporter linked to cotE and spoIVA, the studies on gene expression and localization of the expressed protein in spore has been attempted (Webb et al., 1995; Lewis et al., 1996). The publications disclose that the fusion protein expressed is found in spore by means of observation under fluorescence microscope using fluorescence of GFP; however, they never describe if the fusion protein is displayed and linked on spore surface.

As another example of spore surface display using coat protein, U.S. Pat. No. 5,800,821 discloses a spore as delivery system of antigen. However, the publication does not disclose that the antigen expressed is displayed on

spore surface and the spore containing antigen administered can induce immunization reaction in host.

The present inventors have recognized the shortcomings of the conventional arts described above and developed an efficient and optimized system for spore surface display, which have been confirmed by enzymological, immunological and physiochemical methods using various spore coat proteins.

In one aspect of this invention, there is provided a method for displaying a protein of interest on spore surface, which comprises the steps of: (i) preparing a vector for spore surface display comprising a gene construct containing a gene encoding spore coat protein and a gene encoding a protein of interest, wherein, when expressed, the gene construct expresses a fusion protein between the spore coat protein and the protein of interest; (ii) transforming a host cell with the vector for spore surface display; (iii) displaying the protein of interest on a surface of a spore of the host cell; and (iv) recovering the spore displaying on its surface the protein of interest.

In another aspect of this invention, there is provided a method for improving a protein of interest, which comprises the steps of: (i) constructing a gene library of the protein of interest; (ii) preparing a vector by linking the gene

library to a gene encoding spore coat protein; (iii) transforming a spore-forming host cell with the vector; (iv) forming a spore in the transformed host cell and displaying the protein of interest on a surface of the spore; (v) recovering the spore displaying on its surface the protein of interest; and (vi) screening the spore displaying a variant of the protein of interest having a desired property.

In still another aspect of this invention, there is provided a method for improving a protein of interest using a resistance property of spore, which comprises the steps of: (i) constructing a gene library of the protein of interest; (ii) preparing a vector by linking the gene library to a gene encoding spore coat protein; (iii) transforming a spore-forming host cell with the vector; (iv) forming a spore in the transformed host cell and displaying the protein of interest on a surface of the spore; (v) treating the spore displaying on its surface the protein of interest with one or more selected from the group consisting of organic solvent, heat, acid, base, oxidant, dryness, surfactant and protease; (vi) recovering the spore displaying on its surface the protein of interest; and (vii) screening the spore displaying a variant of the protein of interest having a resistance to the treatment.

In further aspect of this invention, there is provided a method for bioconversion, which comprises the steps of: (i) preparing a vector for spore surface display comprising a gene construct containing a gene encoding spore coat protein and a gene encoding a protein of interest conducting a bioconversion reaction, wherein, when expressed, the gene construct expresses a fusion protein between the spore coat protein and the protein of interest; (ii) transforming a host cell with the vector for spore surface display; (iii) displaying the protein of interest on a surface of a spore of the host cell; (iv) recovering the spore displaying on its surface the protein of interest; and (v) performing the bioconversion reaction using the spore displaying on its surface the protein of interest.

In still further aspect of this invention, there is provided a method for preparing protein microarray, which comprises the steps of: (i) preparing a vector for spore surface display comprising a gene construct containing a gene encoding spore coat protein and a gene encoding antibody or antigen having binding affinity to a protein to be analyzed, wherein, when expressed, the gene construct expresses a fusion protein between the spore coat protein and the antibody or antigen; (ii) transforming a host cell with the vector for spore surface display; (iii) displaying

the antibody or antigen on a surface of a spore of the host cell; (iv) recovering the spore displaying on its surface the antibody or antigen; and (v) immobilizing onto a solid surface the spore displaying on its surface the antibody or antigen.

In another aspect of this invention, there is provided a method producing an antibody to antigen in vertebrates, which comprises the steps of: (i) preparing a vector for spore surface display comprising a gene construct containing a gene encoding spore coat protein and a gene encoding the antigen, wherein, when expressed, the gene construct expresses a fusion protein between the spore coat protein and the antigen; (ii) transforming a host cell with the vector for spore surface display; (iii) displaying the antigen on a surface of a spore of the host cell; (iv) recovering the spore displaying on its surface the antigen; and (v) administering to vertebrates a composition containing an immunologically effective amount of the spore displaying on its surface the antigen.

In still another aspect of this invention, there is provided a method for preparing a whole cell absorber, which comprises the steps of: (i) preparing a vector for spore surface display comprising a gene construct containing a

gene encoding spore coat protein and a gene encoding a protein having a binding affinity to a certain substance, wherein, when expressed, the gene construct expresses a fusion protein between the spore coat protein and the protein; (ii) transforming a host cell with the vector for spore surface display; (iii) displaying the protein on a surface of a spore of the host cell; (iv) recovering the spore displaying on its surface the protein; and (v) immobilizing onto a carrier the spore displaying on its surface the protein.

According to preferred embodiments of this invention, the gene encoding spore coat protein is derived from a spore-forming Gram negative bacterium including *Myxococcus*; a spore-forming Gram positive bacterium including *Bacillus*; a spore-forming *Actionmycete*; a spore-forming yeast including *Saccharomyces cerevisiae*, *Candida* and *Hansenulla* or a spore-forming fungus, but not limited to. More preferably, the gene encoding spore coat protein is derived from a spore-forming Gram positive bacterium, most preferably, *Bacillus* including *Bacillus subtilis* and *Bacillus polymyxa*, etc.

The gene of spore coat protein useful in this invention includes cotA, cotB, cotC, cotD (W. Donovan et al., *J. Mol. Biol.*, 196:1-10(1987)), cotE (L. Zheng et al., *Genes & Develop.*, 2:1047-1054(1988)), cotF (S. Cutting et al., *J.*

Bacteriol., 173:2915-2919(1991)), cotG, cotH, cotJA, cotJC, cotK, cotL, cotM, cotS, cotT (A. Aronson et al., *Mol. Microbiol.*, 3:437-444(1989)), cotV, cotW, cotX, cotY, cotZ (J. Zhang et al., *J. Bacteriol.*, 175:3757-3766(1993)), spoIVA, spoVID and sodA, but not limited to.

In addition, the gene encoding spore coat protein useful in this invention is a modified form or a recombinant form of one selected from the group consisting of cotA, cotB, cotC, cotD, cotE, cotF, cotG, cotH, cotJA, cotJC, cotK, cotL, cotM, cotS, cotT, cotV, cotW, cotX, cotY, cotZ, spoIVA, spoVID and sodA, in which the modified form or the recombinant form has a more compatibility for spore surface display relative to wild type genes. The modified form of the gene is obtained by DNA shuffling method (Stemmer, *Nature*, 370: 389-391(1994)), StEP method (Zhao, H., et al., *Nat. Biotechnol.*, 16: 258-261 (1998)), RPR method (Shao, Z., et al., *Nucleic acids Res.*, 26: 681-683 (1998)), molecular breeding method (Ness, J. E., et al., *Nat. Biotechnol.*, 17: 893-896 (1999)), ITCHY method (Lutz S. and Benkovic S., *Current Opinion in Biotechnology*, 11: 319-324 (2000)), error prone PCR (Cadwell, R. C. and Joyce, G. F., *PCR Methods Appl.*, 2: 28-33 (1992)), point mutagenesis (Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, N. Y., 1989), nucleotide mutagenesis (Smith M. *Annu. Rev. Genet.* 19: 423-462 (1985)), combinatorial cassette

mutagenesis (Wells et al., Gene 34: 315-323 (1985)) and other suitable random mutagenesis.

Further to this, the gene encoding spore coat protein is selected from the group consisting of cotA, cotB, cotC, cotD, cotE, cotF, cotG, cotH, cotJA, cotJC, cotK, cotL, cotM, cotS, cotT, cotV, cotW, cotX, cotY, cotZ, spoIVA, spoVID and sodA, in which the gene has a substituted promoter for its promoter to enhance spore surface display relative to wild type genes. The promoter for enhancing surface display, for example, includes the promoters of cotE or cotG genes, which show higher expression level.

In preferred embodiments of this invention, the gene encoding spore coat protein is selected from the group consisting of cotA, cotE, cotF, cotG, cotH, cotJA, cotJC, cotK, cotL, cotM, cotS, cotT, cotV, cotW, cotX, cotY, cotZ, spoIVA, spoVID and soda, more preferably, cotE or cotG and most preferably, cotG.

According to the present methods, as linking a gene of coat protein and a gene of the protein of interest, the overall sequence, fragments, two or more repeated sequences of the gene of coat protein are useful. In two or more repeated sequences, the repeated sequences may be the same or different each other. The overall sequence, two or more repeated sequences of the gene of the protein of interest are also useful in the fusion sequence. In two or more

repeated sequences, the repeated sequences may be the same or different each other. Other combinations also may be useful in the fusion sequence.

It is understood by one skilled in the art that the gene construct may exist as plasmid in host cell independently or as integrated form into chromosome of host cell. Additionally, in the gene construct, it is recognized by one skilled in the art that the gene of coat protein may be followed or preceded by the gene of the protein of interest. Integrated form into the counterpart gene may be useful.

It is recognized by one skilled in the art that the expression of the fusion protein between coat protein and protein of interest can be induced by virtue of promoters of coat protein gene and protein of interest or other suitable promoters inducible in host cell

The present methods is applicable to any protein, for example, including enzyme, enzyme inhibitor, hormone, hormone analogue, hormone receptor, signal transduction protein, antibody, monoclonal antibody, antigen, attachment protein, structural protein, regulatory protein, toxin protein, cytokine, transcription regulatory protein, blood clotting protein, plant defense-inducing protein and fragments thereof. The applicable proteins include multimer as well as monomer. The surface display of multimeric proteins has been rarely reported, for instance, the surface

display of alkaline phosphatase in *E. coli*, has resulted the display toward inner part of cell outer membrane (Stathopoulus et al., 1996). β -galactosidase used as reporter enzyme in Examples of the present invention must form tetramer to exhibit its activity and has not been published to be successful in surface display. β - galactosidase generally cannot pass across cell membrane and comprises an amino acid sequence detrimental to cell membrane, as a result, the fusion protein between surface display motif and β -galactosidase has been recognized not to be displayed on cell surface. Therefore, the surface display of β -galactosidase described in Examples proves to be surprising.

The term used herein "protein" refers to molecule consisting of peptide bond, for example including oligopeptide and polypeptide.

The host cell suitable in this invention, includes spore-forming Gram negative bacterium including *Myxococcus*, a spore-forming Gram positive bacterium including *Bacillus*, a spore-forming *Actionmycete*, a spore-forming yeast and a spore-forming fungus, but not limited to. Preferably, the host cell is a spore-forming Gram positive bacterium, more preferably, *Bacillus*. In particular, *Bacillus subtilis* is advantageous in the senses that genetic knowledge and experimental methods on its spore forming as well as

culturing method are well known.

According to the present methods, the spore may be reproductive or non-reproductive. In the method for improving a protein, the recovered coats are subject to reproduction but the methods using a spore as delivery means of protein of interest obviate the necessity for reproduction of spore. It is considerable that the organisms genetically engineered is likely to be regulated under laws and rules; hence non-reproductive spore is preferable. For example, *Bacillus subtilis* lack of cwlD gene is preferably used due to being non-reproductive.

According preferred embodiments of this invention, the recovery of spore is performed in such a manner that the display of the protein of interest on the spore surface is maximized by controlling culture time, after which culturing is terminated and the spore is then recovered. Suitable culture time is varied depending upon the type of cell used, for example, in case of using *Bacillus subtilis* as host, the culture time of 16-25 hours is preferred.

In the present methods, the recovery of spore may be carried out according to the conventional methods known to one skilled in the art, more preferably, renografin gradients methods (C. R. Harwood, *et al.*, "Molecular Biological Methods for Bacillus." John Wiley & Sons, New York, p.416(1990)).

As demonstrated in Examples, the stability of spore displaying the foreign protein of interest on its surface is very high in the present invention, indicating maintenance of the integrity of spore surface structure formed by cooperation of coat proteins while the foreign protein is displayed.

The protein of interest displayed on spore surface according to the present methods can be demonstrated with a wide variety of methods as follows: 1) A primary antibody is bound to the protein of interest displayed on spore surface and then reacted with a secondary antibody labeled with fluorescent chemical to stain the spore, followed by observation with fluorescence microscope or analysis with flow cytometry. 2) The protein of interest displayed on spore surface is treated with protease, followed by measurement of the activity of the protein or detecting lower signal with fluorescence microscope or flow cytometry. 3) In case that the protein of interest uses a substrate with higher molecular weight, the direct measurement of the activity of the protein can provide the level of display since the substrate cannot pass across outer coat of spore.

In the method for improving protein, the construction of gene library for the protein of interest is performed by a mutagenesis of the gene encoding the protein of interest of

wild type, in which the mutagenesis includes DNA shuffling method (Stemmer, *Nature*, 370: 389-391(1994)), StEP method (Zhao, H., et al., *Nat. Biotechnol.*, 16: 258-261 (1998)), RPR method (Shao, Z., et al., *Nucleic acids Res.*, 26: 681-683 (1998)), molecular breeding method (Ness, J. E., et al., *Nat. Biotechnol.*, 17: 893-896 (1999)), ITCHY method (Lutz S. and Benkovic S., *Current Opinion in Biotechnology*, 11: 319-324 (2000)), error prone PCR (Cadwell, R. C. and Joyce, G. F., *PCR Methods Appl.*, 2: 28-33 (1992)), point mutagenesis (Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, N. Y., 1989), nucleotide mutagenesis (Smith M. *Annu. Rev. Genet.* 19: 423-462 (1985)), combinatorial cassette mutagenesis (Wells et al., Gene 34: 315-323 (1985)) and other suitable random mutagenesis.

In the method for improving protein, the screening is performed in a rapid manner by means of measuring an activity of the protein or flow cytometry (Georgiou, 2000). In case of using an activity of the protein, the screening is carried out by measuring growth of host expressing the protein or colorimetrical reaction catalyzed by the protein. In the method for improving protein using a resistance property of spore, the screening is carried out in a rapid manner by virtue of measuring an activity of the protein or using the structural stability of the protein.

The methods for improving protein provide in a high-

throughput manner, from wild type, 1) enzymes catalyzing non-biological reaction (e.g., Diels-Alder condensation), 2) enzymes with non-natural steroselectivity or regioselectivity, 3) enzymes with activity in organic solvent or organic solvent-aqueous solution two-phase system, and 4) enzymes with activity in extreme conditions such as high temperature or pressure.

In addition, to select a variant of antibody with enhanced binding affinity, it is general that pH is abruptly changed or the concentration of base is adjusted to elute the variant. In a method using phage or bacteria as carrier, such elution conditions are likely to decrease the viability of phage or bacteria in medium. However, the methods for improving protein using system of spore surface display overcome the drawback.

In the meantime, the bioconversion process using surface-displayed enzymes requires a physiochemical stability of surface displaying host in extreme conditions because the process is usually executed in high temperature and/or organic solvent. In particular, a chemical synthesis valuable in current industry is mainly carried out in organic solvent and the synthesis of chiral compound or the resolution of racemic mixture is also performed in highly severe physiochemical conditions. Therefore, the surface-displayed enzyme as well as the organisms displaying enzyme

is compelled to have stability in such extreme conditions. In this connection, it is demonstrated that the methods for bioconversion using system for spore surface display is largely advantageous.

The chemical processes using surface-displayed enzymes have been proposed (Georgiou et al., 1993). However, the proposed processes have generally required immobilization of cell surface with cross-linking agent since the host displaying enzyme is very unstable during process (Freeman et al., 1996). The present bioconversion process is free from the disadvantage mentioned above. Because the surface-displayed enzyme as well as the host displaying enzyme is largely stable, the present method avoids the immobilization. In Examples described hereinafter, the bioconversion reaction with β-galactosidase is exemplified and thus it is understood by one skilled in the are that the present method can be also applied to any type of enzyme such as lipase, protease, cellulase, glycosyltransferase, oxidoreductase and aldolase. In addition, the present method is useful in single step or multi-step reaction and in aqueous or non-aqueous solution. The present bioconversion method employs spore as free or immobilized form and can be performed with other microbes or enzymes.

Similar to DNA microarray, a protein microarray provides means for analyzing expression or expression level of target

protein in certain cell. In order to fabricate protein array, the suitable proteins to be arrayed must be obtained and then immobilized on solid surface. During analysis using protein array, washing step is necessarily performed to remove unbound proteins and various treatments such as high temperature, higher salt concentration and pH adjustment are executed; therefore, it is pivotal to guarantee proteinaceous substance with higher stability in such detrimental environment.

In addition, the conventional process for preparing protein array needs tedious and repetitive works such as cloning genes of several thousands to tens of thousands of proteins and immobilizing of the proteins expressed. Therefore, there remains a need to improve simplicity and rapidity of the works.

According to the method for preparing protein microarray of this invention, it is ensured that the works described-above cane be performed with much greater readiness. In the present method, a gene construct containing a gene encoding spore coat protein and a gene encoding the desired protein is introduced into host cell and the spore displaying on its surface the desired protein is isolated, followed by immobilization of the isolated spore onto a solid surface. In the method for preparing protein array, the conventional steps may be used (see WO 0061806, WO 0054046, US 5807754,

EP 0818467, WO 9742507, US 5114674 and WO 9635953). The protein microarray manufactured by the present invention has a variety of applicable fields including diagnosis, analysis of gene expression, analysis of interaction between proteins, analysis of interaction between protein and ligand, study on metabolism, screening novel or improved enzymes, combinatorial biochemical synthesis and biosensor.

The solid substrate suitable in the present method includes, but not limited to, glasses (e.g., functionalized glasses), Si, Ge, GaAs, GaP, SiO, SiN_4, modified silicone nitrocellulose, polyvinylidene fluoride, polystylene, polytetrafluoroethylene, polycarbonate, nylon, fiber and combinations thereof. The spore optionally may be attached to the array substrate through linker molecules. It is preferred that the regions of the array surface not being spotted are blocked. The amount of spores applied to each spot (or address) depends on the type of array. Interaction between the protein displayed on spore attached to solid substrate and the sample applied can be detected based on their inherent characteristics (e.g., immunogenicity) or can be rendered detectable by being labeled with an independently detectable tag (e.g., fluorescent, luminescent or radioactive molecules, and epitopes). The data generated with protein array of this invention can be analyzed using known computerized systems such as "reader" and "scanner".

According to the method producing an antibody of this invention, a composition containing an immunologically effective amount of the spore, preferably, further comprises adjuvant such as incomplete and complete Freund's adjuvants. In the present method, the mode of administration is, preferably, injection and more preferably, intravenous, intraperitoneal, subcutaneous and intramuscular injections. Boosting within suitable period after the first administration is preferable to yield a sufficient amount of antibody.

Meanwhile, in the process for preparing absorption chromatography, antibody or polypeptide is produced, purified and immobilized on a carrier. Generally, it is very difficult to prepare the bioabsorbers. The disadvantage may be overcome using whole cell displaying protein as described in Georgiou et al., 1997. Therefore, the system for spore surface display of this invention provides a whole cell absorber to solve the problems of the known absorbers.

In further aspect of this invention, there is provided a microbial transformant for spore surface display of a protein of interest, characterized in that the transformant is produced by transformation with a vector for spore surface display containing (i) a gene encoding a protein of interest and (ii) a gene encoding spore coat protein is

selected from the group consisting of cotA, cotB, cotC, cotD, cotE, cotF, cotG, cotH, cotJA, cotJC, cotK, cotL, cotM, cotS, cotT, cotV, cotW, cotX, cotY, cotZ, spoIVA, spoVID and sodA, in which when expressed, a fusion protein between the spore coat protein and the protein of interest is expressed.

According to preferred embodiment, the transformant is derived from a variant mutated to enhance spore surface display. For example, the mutation to enhance spore surface display eliminates a production of extracellular secretory protease in the transformant, so that the protein of interest displayed on spore surface is stably maintained. In addition, the mutation to enhance spore surface display eliminates a production of intracellular protease in the transformant. It is also preferred that a gene or genes involved in spore forming is subject to mutation in order to the rate of spore forming (Perego, M., et al., *Mol. Microbiol.* 19: 1151-1157 (1996)).

In still further aspect of this invention, there is provide a spore for spore surface display of a protein of interest, characterized in that the spore displays the protein of interest on its surface.

According to the present invention, the spore may be reproductive or non-reproductive one which is selected based on its application field. Preferably, the non-reproductive

spore can be obtained by virtue of one or more methods selected from the group consisting of genetic method (Popham D. L., et al., *J. Bacteriol.*, 181: 6205-6209 (1999)), chemical method (Setlow T. R., et al., *J. Appl. Microbiol.*, 89: 330-338 (2000)) and physical method (Munakata N, et al., *Photochem. Photobiol.*, 54: 761-768 (1991)). The genetic method to make the spore non-reproductive is accomplished by, for example, deleting a gene of host cell involved in reproduction of spore.

In the present invention, it is preferred that the spore is derived from a variant mutated to increase its agglutination property because in bioconversion performed in industrial scale, the separation between the resulting product and spores is rendered easier. The increase of the agglutination property in the spore is accomplished by one or more methods selected from the group consisting of genetic method, chemical method and physical method. As example of the physical method, the heat treatment can be proposed (Wiencek K. M., et al., *Appl. Environ. Microbiol.*, 56: 2600-2605 (1990)).

In another aspect of this invention, there is provided a vector for spore surface display, characterized in that the vector comprises a replication origin, an antibiotic-resistance gene, a restriction site, a gene encoding a spore

coat protein, a gene encoding a protein of interest and a promoter operatively linked to the gene encoding a spore coat protein, in which when expressed, a fusion protein between the spore coat protein and the protein of interest is expressed.

According to preferred embodiment, the gene encoding a spore coat protein is selected from the group consisting of cotA, cotB, cotC, cotD, cotE, cotF, cotG, cotH, cotJA, cotJC, cotK, cotL, cotM, cotS, cotT, cotV, cotW, cotX, cotY, cotZ, spoIVA, spoVID and sodA, more preferably, cotE or cotG, and most preferably, cotG.

In the vector of this invention, the replication origin can include various origins known to one skilled in the art, for example, when the vector is introduced into a spore-forming yeast, 2µ , ARS, ARS1 or ARS2 can be used as replication origin. In case of using *Bacillus* as host, ori 322, ColE1 origin, Rep1060, etc. can be used. The antibiotic-resistance gene used as selective marker, when prokaryote such as *Bacillus* is used as host, is a resistance gene to antibiotics acting to prokaryotes, for example, including kanamycin, ampicillin, carbenicillin, chloramphenicol, streptomycin, geneticin, neomycin and tetracycline. The promoter used in the present vector includes a promoter of the gene of spore coat protein and a known promoter operable in host cell.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a microscopic photograph showing spores of *Bacillus subtilis* purified according to method described in U.S. Pat. No. 5,766,914;

Fig. 2 is a microscopic photograph showing spores of *Bacillus subtilis* purified according to renografin gradients method;

Fig. 3 is a genetic map of the recombinant vector pCotE-lacZ of the present invention;

Fig. 4 is a genetic map of the recombinant vector pCotG-lacZ of the present invention;

Fig. 5 represents screening results demonstrating the preferred surface display motif in the present invention;

Fig. 6 is a graph showing the affect of protease to β-galactosidase displayed on spore surface;

Fig. 7 is a graph showing the activity of β-galactosidase displayed on spore surface in accordance with culture time;

Fig. 8 is a graph representing the heat stability of *Bacillus subtilis* DB104 strain displaying on its surface the protein;

Fig. 9 is a genetic map of recombinant vector pCSK-cotG-CMCase of this invention;

Fig. 10 is a graph showing analysis of spore surface-

displayed carboxymethylcellulase using flow cytometry;

Fig. 11 is a graph showing analysis of spore surface-displayed levansucrase using flow cytometry;

Fig. 12 is a graph showing the activity of spore surface-displayed levansucrase;

Fig. 13 is a graph representing analysis of spore surface-displayed monoclonal antibody using flow cytometry;

Fig. 14 is a graph demonstrating selectivity to spore displaying single chain Fv;

Fig. 15 is a graph representing analysis with flow cytometry of monoclonal antibody library to have binding affinity to Pre-S region of hepatitis B virus;

Fig. 16 is a graph showing analysis of spore surface-displayed GFP using flow cytometry; and

Figs. 17a to 17d are graphs representing isolation with flow cytometry of spores displaying improved GFP.

The following specific examples are intended to be illustrative of the invention and should not be construed as limiting the scope of the invention as defined by appended claims.

EXAMPLES

Example I: Isolation of the Gene Encoding Coat Proteins

I-1: <u>Construction of the Vector for Spore Surface Display</u>

To isolate the most appropriate coat protein for spore surface display among coat proteins consisting of spore, the recombinant vector having the gene encoding a fusion protein between coat protein and β-galactosidase was constructed as follow:

To begin with, the DNA was extracted from the *Bacillus subtilis* 168 strain provided from Dr. F. Kunst (Kunst F., et al., *Nature*, 390: 249-256(1997)) by Kalman's method (Kalman S., et al., *Appl. Environ. Microbiol.* 59, 1131-1137(1993)), and the purified DNA was served as template for PCR to spoIVA primers (SEQ ID NOs: 1 and 2), cotB primers (SEQ ID NOs: 3 and 4), cotC primers (SEQ ID NOs: 5 and 6), cotD primers (SEQ ID NOs: 7 and 8), cotE primers (SEQ ID NOs: 9 and 10), cotG primers (SEQ ID NOs: 11 and 12), cotH primers (SEQ ID NOs: 13 and 14), cotM primers (SEQ ID NOs: 15 and 16), cotV primers (SEQ ID NOs: 17 and 18), cotX primers (SEQ ID NOs: 19 and 20) and cotY primers (SEQ ID NOs: 21 and 22). Taq polymerase purchased from Boehringer Mannheim was used for total 35 cycles of PCR under condition of denaturation for 30 sec at 94℃, annealing for 30 sec at 55℃ and extension for 1 min at 72℃.

After then, each amplified PCR products were digested with *Bam*HI and *Sal*I and cloned between *Bam*HI and *Sal*I sites of plasmid pDG1728 which is a gratuitous gift by Dr. P. Stragier (Geurout-Fleury, A.M., et al., *Gene*, 180: 57-

61(1996)), thus the constructed vectors express the fusion protein of coat protein and β-galactosidase. Fig. 3a shows the genetic map of pCotE-lacZ expressing fusion protein of CotE protein and β-galactosidase and Fig. 3b shows the genetic map of pCotG-lacZ expressing fusion protein of CotG protein and β-galactosidase.

SEQ ID NO:23 shows the sequence of cotE-lacZ fused genes and SEQ ID NO:24 shows the amino acid sequence of CotE-LacZ fusion protein. In SEQ ID NO:23, promoter for cotE is 1-329, CotE structural gene is 330-872, restriction site is 873-878 and LacZ structural gene is 879-3902.

SEQ ID NO:25 shows the sequence of cotG-lacZ fused genes and SEQ ID NO:26 shows the amino acid sequence of CotG-LacZ fusion protein. In SEQ ID NO:25, promoter of cotG is 1-460, CotE structural gene is 461-1045, restriction site is 1046-1051 and LacZ structural gene is 1052-4075.

I-2: Pure Isolation of Spores

Constructed recombinant expression vectors were transformed into *Bacillus subtilis* DB104 (Kawamura F. and Doi R.H., J. Bacteriol. 160: 442-444(1984)) using natural transformation (C.R. Harwood, et al., Molecular Biological Methods for Bacillus, John Wiley & Sons, New York, p.416(1990)).

Other methods such as conjugation or trnasduction can be

applied for introduction of the recombinant vectors into *Bacillus* strain.

Subsequently, each *Bacillus* strain comprising the fused gene between coat protein and β-galactosidase was cultured for 24 hr at a shaking incubator (37℃, 250 rpm) in GYS medium ($(NH_4)_2SO_4$ 2 g/l, Yeast extract 2 g/l, K_2HPO_4 0.5 g/l, glucose 1 g/l, $MgSO_4 \cdot 5H_2O$ 0.07 g/l), and the only pure spores were isolated using renografin gradients method (C. R. Harwood, et al., "Molecular Biological Methods for Bacillus." John Wiley & Sons, New York, p.416(1990)).

I-3: <u>Display of Proteins on Spore Surface</u>

The spores isolated in the above-described Example and the cell pellet of *Bacillus subtilis* DB104 were subjected to evaluation of the activity of β-galactosidase using Miller's method (Miller, "Experiments in Molecular Genetics", Cold Spring Harbor, NY: Cold Spring Harbor Laboratory, p.352-355(1972)) and the results are shown in Fig. 5. In Fig. 5, the gray bar indicates cell pellet, the black bar indicates the activity of β-galactosidase in purely isolated spores and '1' relates to result of control *Bacillus subtilis* DB104; '2' to result of SpoIVA-LacZ; '3' to result of CotB-LacZ; '4' to result of CotC-LacZ; '5' to result of CotD-Lacz; '6' to result of CotE-LacZ; '7' to result of CotG-LacZ; '8' to result of CotH-LacZ; '9' to result of CotM-

LacZ; '10' to result of CotV-LacZ; '11' to result of CotX-LacZ; and '12' to result of CotY-LacZ fusion protein, respectively.

As shown in Fig. 5, it is known that Deits TL (U.S. Pat. No. 5,766,914) fails to induce the sufficient surface display of cotC and cotD since the expression levels of cotC and cotD are as low as the control. However, the expression level of cotE and cotG are comparatively high and especially, expression level of cotG is remarkably high comparing to other coat proteins. In addition, in the isolated spores, the surface display using cotG shows the highest enzyme activity, which demonstrates that CotG-LacZ fusion proteins are the highest level of display on spore surface.

Considering the expression level and the amount of fusion proteins displayed on spore surface, it is known that the cotG is the most preferable surface display motif. It is known to one skilled in the art that these results exclude other coat proteins other than cotG from applying to spore surface display.

I-4: <u>Effect of Proteases on the Surface-Displayed Enzymes</u>

To confirm whether the surface-displayed β-galactosidase is degraded or not, the purely isolated spore displaying CotG-LacZ was resuspended into 100 μl of PBS solution, and

each 10 mg/mℓ of protease K, protease type XIV or trypsin was treated. Thereafter, the activity of β-galactosidase was measured as described above and the results are shown in Fig. 6. As shown in Fig. 6, the activity of spore surface-displayed β-galactosidase is decreased with some variations in each result. These results give the evidence for the localization of β-galactosidase on spore surface.

DB104 strain lacking neutral and alkaline protease and WB700 strain (Ye, R., et al., *Biotechnology and Bioengineering*, 62:87-96(1999)) lacking 7 proteases among proteases secreted from *Bacillus subtilis* were transformed with the pCotG-lacZ expression vector using natural transformation method as described in example I-1, and the activity of β-galactosidase in cell pellet and spores was measured as described in example I-3 (Fig. 7). As shown in Fig. 7, while the enzyme activity is abruptly decreased in DB104 strain as time goes, WB700 strain shows slight decrease in enzyme activity. These results indicate that the displayed β-galactosidases on spore surface are degraded in DB104 strain by the proteases secreted extracellularly; however, the displayed β-galactosidases in WB700 are stably maintained because of lack of the proteases secreted extracellularly. Therefore, the results also support the localization of β-galactosidase on spore surface.

Example II: Spore Production Depending on Culture Time

As shown in Fig. 7, it is required to stop incubation on a specific time point and isolate spores. In DB104, the enzyme activity of spores after 38 hr of incubation is significantly low comparing to that after 24 hr of incubation. Thus, it is demonstrated that the adjustment of incubation time makes it possible to yield spores displaying enzyme on its surface with the greatest enzyme activity.

Example III: Characterization of Spores Dispalying β-galactosidase

Heat resistance was measured as follow in spores displaying β-galactosidase: 100 μl of spores isolated by renografin gradients in Example I-2 were heated for 15 min and then spread on LB plates to evaluate viability of spores (Fig. 8). As shown in Fig. 8, spores displaying CotG-LacZ show similar heat resistance to spores without surface protein. In a result, the display of the foreign protein fused to coat protein on spore surface does not affect on its inherent characteristics such as heat resistance. Moreover, these results provide the promising usage of spore displaying on its surface enzyme in chemical reactions at high temperature. In addition, from these results, it is suggested that the spores transformed according to the present invention remain their inherent resistances to

lysozyme, a bacterial cell wall-degrading enzyme and solvent.

Example IV: Displaying Various Enzymes on Spore Surface

IV-1: Construction of Recombinant Vectors

To use spores displaying various enzymes, it is prerequisite to confirm whether various enzymes in addition to β-galactosidase can be surface-displayed. Firstly, plasmid pHPS9 (Haima, et al., *Gene*, 86:63-69(1990)) was digested by *Eco*RI and *Hin*dIII and manipulated into blunt ends using Klenow enzyme. Then, DNA fragment containing multiple cloning sites, which was obtained from plasmid p123T (EMBL Z46733) with *Bss*HII, was ligated to the blunt-ended pHPS9 plasmid to use as virgin vector named pCSK1 in the following experiments. The pCSK-cotG plasmid was prepared by restricting pCSK1 plasmid with *Bam*HI and *Pst*I and ligating PCR-amplified cotG gene. In the course of PCR for cotG gene amplification, a linker between cotG gene and target gene was incorporated using cotG-linker 5 primer (SEQ ID NO:27) and 3 primer (SEQ ID NO:12) with template of DNA in *Bacillus subtilis*.

In other experiments, genes encoding carboxymethyl cellulase, levansucrase and lipase was prepared as follows: Carboxymethyl cellulase cloned in pBSI plasmid (S. H. Park et al., Agric. Biol. Chem., 55: 441-448(1991)) was directly

employed. The pBS1 plasmid contains the gene encoding carboxymethylcelluase cloned from *Bacillus subtilis* BSE616 strain. In the present Example, PCR was performed with the pBS1 as template using primer represented by SEQ ID NOs:28 and 29. In the case of PCR for levansucrase, pSSTS110 plasmid (Jung, H.-C., et al., *Nat. Biotech.*, 16; 576-580(1998)) was used as template and primers represented by SEQ ID NOs:30 and 31 were used. In PCR for lipase, pTOTAL (Ahn, J.-H., et al., *J. Bacteriol.*, 181: 1847-1852(1999)) was added as template and primers of SEQ ID NOs: 32 and 33 were used. All PCRs were performed in the same condition as described in Example I-1.

Recombinant vectors containing gene coding for fusion between CotG and the carboxymethylcelluase, levansucrase or lipase were prepared by cloning into pCSK-cotG using *Pst*I and *Bam*HI restriction enzymes both in vector and in the PCR-amplified inserts. As an example of the above construction, Fig. 9 shows pCSK-cotG-CMCase which is the recombinant vector encoding fusion protein between CotG and carboxymethylcellulase. Transformed *Bacillus subtilis* DB104 with pCSK-cotG-CMCase was named *Bacillus subtilis* GFSD18 and deposited at the Korean Collection for Type Cultures (KCTC, KR) with accession No. KCTC 0887BP (November 16, 2000).

SEQ ID NO:34 shows nucleotide sequence of fused cotG-CMCase genes and SEQ ID NO:35 shows amino acid sequence of

CotG-CMCase encoded by SEQ ID NO:34. In SEQ ID NO:34, promoter for cotG is 1-460, structural gene for CotG is 461-1045, linker is 1046-1084, and structural gene for CMCase is 1085-2491.

IV-2: <u>Expression of Recombinant Vectors and Verification</u>

The above-prepared recombinant vectors were employed for transformation of *Bacillus subtilis* DB104 with the same procedures as described in Example I-2. Subsequently, each transformed *Bacillus* strains was cultured for 24 hr at a shaking incubator (37℃, 250 rpm) in GYS midium, the only pure spores were isolated using renografin gradients method, and enzyme activity of carboxymethylcellulase (Kim, et al., *Appl. Environ. Microbial.*, 66:788-793(2000)), levansucrase (Jung, et al., *Nat. Biotech.*, 16:576-580(1998)) or lipase was evaluated. The activity of lipase was evaluated as follow: The spores suspended in 10% PBS was mixed with 10% olive oil, reacted for 48 hr, treated with 0.2 mℓ cupric acid on supernatant solution and the observance of OD was performed at 715 nm.

In the case of carboxymethylcellulase, the activity of enzyme displayed on spore was 175 mU comparing to 0 mU in control. In other verifying method, carboxymethylcellulase-specific andtibody (Kim, et al., *Appl. Environ. Microbiol.*, 66:788-793(2000)) was probed for flow cytometry (FACSort,

Becton Dickinson, USA) and the carboxymethylcellulases were detected on the surface of spores transformed by pCSK-cotG-CMCase (Fig. 10).

The activity of levansucrase was also high in spores transformed by recombinant vector (Fig. 12) and the levansucrases were detected on the surface of transformed spores as verified with flow cytometery using levan sucrase-specific antibody (Jung, et al., *Nat. Biotech.*, 16:576-580(1998)) in the same procedures as above-described in carboxymethylcellulase (Fig. 11).

The activity of lipase was measured as A_{715} = 0.14 in spores transformed with recombinant vector.

On the basis of these results, it is demonstrated that various enzymes as well as β-galactosidase can be displayed on the surface of spore according to the present invention.

Based on the results in these examples and example I, it is known to one skilled in the art that the gene construct containing gene encoding fusion protein between coat protein and protein of interest may exist as plasmid in host cell independently or as integrated form into chromosome of host cell and both forms may lead to successful spore surface display. It is also recognizable that the gene of coat protein may be followed or preceded by the gene of the protein of interest. In addition, it is recognized that in

the gene construct, the overall sequence, fragments, two or more repeated sequences of the gene of coat protein are useful. In two or more repeated sequences, the repeated sequences may be the same or different each other. The overall sequence, two or more repeated sequences of the gene of the protein of interest are also useful in the fusion sequence. In two or more repeated sequences, the repeated sequences may be the same or different each other.

It is recognized by one skilled in the art that the expression of the fusion protein between coat protein and protein of interest can be induced by virtue of promoters of coat protein gene and other suitable promoters operable in host cell. Any vector carrying the present gene construct may be used in this invention, which is recognized by one skilled in the art referring to these results.

It is known that both monomric and multimeric enzyme can be applied for the present invention since the β-galactosidase used in example I is tetramer (U. Karlsson et al., *J. Ultrastruct. Res.*, 10:457-469(1964)) and the enzymes described in this Example are monomers.

Example V: Display of Antibody on Spore Surface and Screening for Directed Evolution

On the purpose of application of other proteins in addition to enzymes, the experiment to display antibody on

spore surface was performed as follows:

V-1: Construction of Recombinant Vector for Surface Display of Single Chain Fv

Gene encoding single chain Fv against Pre-S2 domain (SEQ ID NO:36) of hepatitis B virus (HBV) was linked to cotG gene encoding surface protein of *Bacillus subtilis* spore. Single chain Fv gene was amplified by PCR with pAScFv101 (WO 9737025) as template and with primers described in SEQ ID NOs:37 and 38. Taq polymerase purchased from Bioneer (Korea) was used for total 30 cycles of PCR under condition of denaturation for 30 sec at 94 ℃, annealing for 30 sec at 55 ℃ and extension for 1 min at 72 ℃. And then, each PCR product was restricted by *Apa*I and *Nhe*I, cloned into pCSK-CotG between the same restriction sites (pCSK-CotG-scFv) and transformed into JM109 using transformation method by Inoue, et al. (Inoue, H., et al., *Gene*, 96:23-28(1990)). The amplified vectors for displaying on spore surface were isolated by alkaline extraction method (Sambrook et al., *Molecular Cloning: A laboratory Manual*, Cold Spring Harbor, N.Y.,1989) and transformed into *Bacillus subtilis* DB104 by natural transformation as described in Example I-1.

V-2: Verification of Single Chain Fv Display on Spore Surface Using Flow Cytometry

Affinity of the displayed single chain Fv against the Pre-S2 of HBV was evaluated by FACSort as the following procedures.

Firstly, Pre-S2 peptide was labeled with fluorescein (PanVera, USA) using fluorescein succinidimyl ester coupling method.

The transformed strains were inoculated into LB broth containing 5 μg/mℓ chloroamphenicol, pre-cultured for 8-10 hr at 37℃, 1% of seed culture was inoculated into GYS broth for sporulation, cultured for 24 hr at 37℃ and the cultured media was harvested. The pure spores were isolated using renografin gradients method, 100 μℓ pure spores were blocked with PBS containing 3% skim milk to inhibit non-specific binding and reacted with 10 μℓ of fluorescein labeled Pre-S2 peptide. Thereafter, the spores bound to fluorescein labeled Pre-S2 peptide were detected in the same procedures as described in example IV (Fig. 13). As shown in Fig. 13, it is demonstrated that the monoclonal antibody against Pre-S2 peptide is successfully displayed without reduction of the affinity to its antigen.

According to the above results, it is recognized that the present methods may be applicable to any protein, for example, enzyme, hormone, hormone analogue, enzyme inhibitor, signal transduction protein or its fragment, antibody or its fragment, antigen protein, attachment protein, structural

protein, regulatory protein, toxin protein, plant defense-inducing protein.

V-3: Selection of Spores Displaying Single Chain Fv using Flow Cytometry

Whether the displayed single chain Fv has affinity to Pre-S2 of HBV was verified with FACSort as follows:

The transformed strains were inoculated into LB broth containing 5 μg/mℓ chloroamphenicol, pre-cultured for 8-10 hr at 37℃, 1% of seed culture was inoculated into GYS broth for sporulation, cultured for 24 hr at 37℃ and the cultured media was harvested. And then, 50 mℓ of harvested culture medium was centrifuged at 10,000 g for 10 min, supernatant was discarded, bacteria were resuspended in 500 μℓ of 20% renografin (Sigma, USA). 100 μℓ of resuspended cell was carefully flowed onto 500 μℓ of 50% renogrant in microtube to form layer, the microtube was centrifuged at 10.000 g for 30 min and pure spores were isolated from pellet.

To discard remained renografin, spores were rinsed 3 times with DW and resuspended in PBS buffer. And then, spores displaying single chain Fv were mixed with wild type spores at a ratio of 1:103 and 1:105 and the spores with affinity to Pre-S2 of HBV were harvested using fluorescein-labeled Pre-S2 peptide and FACSort.

The selectivity was evaluated by colony-forming assay on

LB agar plates and LB agar plates containing 5 μg/mℓ of chloroamphenicol comparing to wild type. Spores displaying surface single chain Fv are resistant to chloroamphenicol owing to chloroamphenicol resistant gene contained in the recombinant vectors.

Fig. 14 shows the selectivity of spores displaying single chain Fv in each ratio (selectivity = ratio of spores displaying single chain Fv after flow cytometry/ ratio of spores displaying single chain Fv before flow cytometry). In the case that the ratio of spores displaying single chain Fv before flow cytometry is 10^{-5}, the selectivity was over 4,000, which indicates that spores with enhanced affinity can be selected by flow cytometry among spores displaying various antibody libraries.

V-4: <u>Directed Evolution of Single Chain Fv Displayed on Spore Surface</u>

To display single chain Fv library on spore surface, the gene encoding single chain Fv against Pre-S2 of HBV was amplified by error-prone PCR. PCR was carried out using pAScFv101 plasmid described in the example V-1 as template and SEQ ID NOs:37 and 38 as primer. PCR mixture was prepared by mixing 0.3 μM of each primers, 5 ng of DNA template, PCR solution (10mM Tris(pH 8.3), 50 mM KCl, 7 mM $MgCl_2$, 0.01% (w/v) gelatin), 0.2 mM dGTP, 0.2 mM dATP, 1 mM dTTP, 1 mM

dCTP, 5 U Taq polymerase from Bioneer (Korea) and DW up to 100 µl. Total 13 cycles of PCR was performed under condition of denaturation for 30 sec at 94℃, annealing for 30 sec at 50℃ and extension for 1 min at 72℃.

Subsequently, restricted PCR products with *Apa*I and *Nhe*I were cloned into pCSK-CotG, vector for displaying on spore surface, between the same restriction sites and library was prepared by transforming the cloned vectors into JM109 *E. coli* with the method of Inoue et al.

The vectors for displaying on spore surface were isolated by alkaline extraction method and transformed into *Bacillus subtilis* DB104 by natural transformation. And then, single chain Fv library against Pre-S2 of HBV was displayed on spore surface as described in example V-2 (Fig. 15).

As shown in Fig. 15, spores with increased fluorescence (i.e., increased affinity) were isolated. This result demonstrates the applicability of the present invention to prepare and select protein variants with improved characteristics.

Example VI: Bioconversion using Spores Displaying Protein of Interest

Forte of transglycosylation by enzyme is the capability of formation of site-specific glycosidic linkage without protection/de-protection step. There have been studied for

formation of glycosidic linkage by 1) induction of reverse hydrolysis in non-aqueous system using glycosidase which is conventionally available glycosidic hydrolyzing enzyme and 2) transglycosylation in which glycosidic linkage is substituted with receptor alcohol instead of hydrolysis of glycosidic linkage by water (G. Ljunger et al., *Enzyme Microb. Technol.*, 16:1808-1814(1994); T. Usui et al., *Carbohytdr. Res.*, 244:315-323(1993); and R. Lopez et al., *J. Org. Chem.*, 59:737-745(1994)). The above conventional methods usually use organic solvent to increase synthetic yield and inhibit hydrolysis. However, because the organic solvent inactivates enzyme, it is difficult to accomplish the high yield. Thus, it is necessary to inhibit the inactivation of glycosidase in organic solvent for higher glycosylation yield.

The purpose of the Example is to exemplify the higher glycosylation yield with improved enzyme stability even in organic solvent by virtue of displaying glycosidase on the surface of hydrophobic *Bacillus* spores.

VI-1: Stability of β-galactosidase Displayed on Surface of Spores in Organic Solvent

Each of β-galactosidase in free form (Sigma, USA) and the β-galactosidase displayed on surface of *Bacillus* spore was dispersed into 500 $\mu\ell$ of Tris-HCl buffer (pH 7.5), added the

same volume of the various solvents described in Table 1, mixed for 37℃ for 1 hr and the remained enzymatic activity was measured by Miller method described in Example I-3 (Table 1).

TABLE 1

	Residual activity (%)	
	Free form β-galactosidase	Surface-Displayed β-galactosidase
Control	100	100
Hexane	84.3	100
Ether	48.2	77.2
Toluene	4.2	51.9
Ethylacetate	0.1	9.6
Acetonitril	0.0	0.8
Ethanol	0.0	0.0

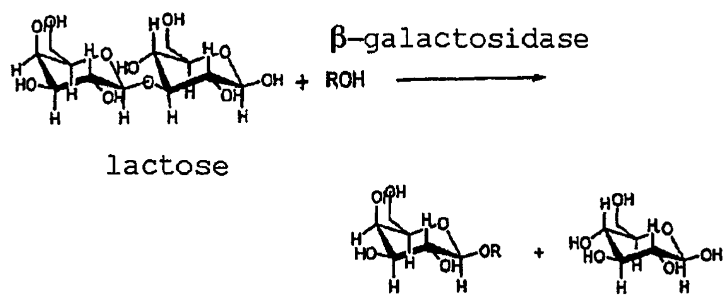
As shown in Table 1, the displayed β-galactosidase shows higher stability than that of free form β-galactosidase in various organic solvents.

VI-2: <u>Transglycosylation Reaction in Water-Organic Solvent Two-phase System Using β-galactosidase Displayed on Spore Surface</u>

To perform transglycosylation in two-phase system, β-galactosidase, which is one of conventional glycosidase, is

used as a model for glycosylation reaction (Scheme 1).

Scheme 1



At first, 1 mℓ of 1 M lactose in 10 mM phosphate buffer (pH 5.1) was mixed with 10 mℓ of 10 mM 5-phenyl-1-pentanol in hexane for reaction solution. And then, β-galactosidase displayed on spore surface (240 U; 1 U = the amount of enzyme capable of hydrolysis of 1 μmol ONPG (*o*-nitrophenyl-β-D-galactopyranoside) for 1 min at 37℃) and free form β-galactosidase (240 U) was added into the above reaction solution, respectively, and reacted for 48 hr at 30℃ while stirring.

In results, the yield of 5-phenylpenthyl-β-D-galtopyranoside was 21% by β-galactosidase displayed on spore surface; however, in free form β-galactosidase, the hydrolysis of lactose only occurred with no transglycosylation. Such result is ascribed to the increased stability, in organic solvent, of β-galactosidase displayed on spore surface. Actually, after 72 hr reaction, about 5% of enzyme activity was detected in the displayed β-

galactosidase while measured the complete inactivation in free form β-galactosidase. Another advantage of the displayed β-galactosidase owes to hydrophobicity of *Bacillus* spores. In other words, the distribution of displayed β-galactosidase at interface between water and organic solvent phase inhibits the hydrolysis comparing to free form β-galactosidase.

Based on the results of this Example, it is understood that variants and modifications thereof falling within the spirit of the invention may become apparent to those skilled in the art, for example, any enzymes in addition to β-galactosidase such as lipase and protease can be employed for bioconversion of the present invention. In addition, the present bioconversion is useful in single step or multi-step reaction and in aqueous or non-aqueous solution. The present bioconversion method can employ spore as free or immobilized form and can be performed with other microbes or enzymes.

Example VII: Display of Antigen on Spore Surface

By displaying antigen on spore surface, antigen capable of inducing immune response *in vivo* can be applied as live vaccine. *Bacillus subtilis* has been considered as safe strain to human body since it has been employed in food fermentation for a long time (Sonenshein A.L., et al.,

Bacillus subtilis and other gram-positive bacteria. *American society for Microbiology*, Washington, p871(1993)).

Gene for CotE-antigen fusion protein is constructed by cloning the gene for surface antigen of HBV into pCotG-lacZ vector constructed in Example I-1. Thereafter, the constructed recombinant vector is transformed into *Bacillus substilis* and the transformants are cultured in GYS medium. And then, the antigen-displaying spores are purely isolated from culture medium by renografin gradients method.

Example VIII: Protein Improvement Using Spore Displaying Protein of Interest

For example of application of the present invention to high-throughput screening of target protein and to protein improvement, GFP (Green Fluorescence Protein) was used as follows:

VIII-1: <u>Construction of Vector for GFP Display on Spore Surface</u>

gfp gene was cloned into pCSK-CotG vector constructed in Example IV-1 and the following sub-cloning procedures were performed for display on spore surface. Each primer was prepared for the purpose of fusing *cotG* gene to EGFP and GFPuv genes. The fluorescence intensity of EGFP (Excit./Emis. Maxima (nm): 488/509; Clontech, USA) has 35-fold stronger

than that of wild type GFP and thus results in detection even in FITC filter and GFPuv (Excit./Emis. Maxima (nm): 395/509; Clontech, USA) is detectable with UV. For further manipulation, *Nhe*I and *Hind*III restriction sites were inserted into primers for *egfp* gene (SEQ ID NOs:39 and 40) and *Pst*I and *Eco*RI restriction sites were inserted in primers for *gfpvu* gene (SEQ ID NOs:41 and 42).

Each of *egfp* (800 bp) and *gfpuv* (720 bp) genes was amplified by PCR (MJ Research PTC-100™ programmable Thermal Controller; 95℃ 30 sec, 55℃ 30 sec, 72℃ 2 min, 25 cycles) using *Pfu* Turbo polymerase (Stratagene, USA) and pEGFP-C1 (Clontedch, USA) or pGFPuv (Clontech, USA) as template.

Thereafter, pCSK-CotG-EGFP or pCSK-CotG-GFPuv vectors were constructed by cloning the restricted PCR products into *Nhe*I/*Hind*III (*egfp* gene) or *Pst*I/*Eco*RI (*gfpuv* gene) restriction sites of pCSK-CotG vector.

VIII-2: <u>Display and Confirmation of GFP on Spore Surface</u>

The constructed vectors were transformed into *Bacillus subtilis* DB104 by natural transformation. Transformants were selected on LB agar plate containing 5 μg/mℓ chloroamphenicol. Through the selection, *Bacillus subtilis* DB104-SDG-EGFP strain for display of EGFP and *Bacillus subtilis* DB104-SDG-GFPuv strain for display of GFPvu on spore surface were obtained. As control strains, *Bacillus subtilis* DB104-SDC

strain transformed with only pCSK vector and *Bacillus subtilis* DB104-SDG strain transformed for expressing only CotG protein were prepared.

For analysis of GFP display on spore surface, the above *Bacillus subtilis* DB104-SDC, -SDG, -SDG-EGFP and -SDG-GFPuv were inoculated into LB broth containing 5 $\mu g/m\ell$ chloroamphenicol and spores were then purified as described in Example V-4.

Subsequently, the display of GFP on spore surface was analyzed by measuring GFP fluorescence with flow cytometry in similar manner to Example IV (Fig. 16). In Fig. 16, curves (1)-(4) indicate the results of spores of DB104-SDC DB104-SDG, DB104-SDG-GFPuv and DB104-SDG-EFGP, respectively.

As shown in Fig. 16, the fluorescent intensity of spores derived from DB1047-SDG-EGFP (recombinant strain for EGFP-spore surface display) and DB104-SDG-GFPuv (recombinant strain for GFPuv-spore surface display) is significantly higher than that of DB104-SDC and DB104-SDG as control. In above results, the successful display of EGFP or GFPuv is validated by noticeable change of peaks indicating fluorescence in spore on its surface displaying EGFP or GFPuv comparing to controls.

VIII-3: <u>Improvement of GFP</u>

For the purpose of GFP improvement, error prone PCR was

performed with template of pGFPuv vector (Clontech, USA) containing *gfpuv* gene using primers of SEQ ID NOs: 42 and 43. PCR mixture was prepared by mixing 0.3 µM of each primers, 5 ng of DNA template, PCR solution (10mM Tris(pH 8.3), 50 mM KCl, 7 mM $MgCl_2$, 0.01% (w/v) gelatin), 0.2 mM dGTP, 0.2 mM dATP, 1 mM dTTP, 1 mM dCTP, 0.15 mM $MnCl_2$, 5 U Taq polymerase from Bioneer (Korea) and DW up to 100 µl. Total 13 cycles of PCR was performed under condition of denaturation for 30 sec at 94℃, annealing for 30 sec at 50℃ and extension for 1 min at 72℃.

Subsequently, the *gfpuv* genes were discarded from pCSK-CotG-GFPuv vectors by restriction with *Pst*I/*Eco*RI, the above PCR-amplified inserts were cloned into the vectors with the same restriction sites and *Bacillus substilis* DB104 was transformed with the cloned vectors by natural transformation to construct *gfpuv* library displayed on spore surface. Then, the prepared library was inoculated into GYS medium for sporulation and pure spores were isolated as described in Example V-4. Transformant spores displaying improved GFP variant were screened by measuring GFP fluorescence with flow cytometry (Figs. 17a to 17d). Figs. 17a to 17d indicates the analysis of flow cytometry from Bacillus subtilis DB104-SDC, DB104-SDG-GFPuv, DB104-SDG-EGFP and DB104-SDG-GFP with *gfp* library subject to error prone PCR, respectively.

To isolate spores with higher fluorescent intensity than spores derived from DB104-SDG-EGFP and DB104-SDG-GFP control strains, the isolation of spores with higher fluorescence (region R1) among spores displaying GFP library was repeated several times.

It is understood that using the above method, the improved GFP protein exhibiting higher fluorescence intensity or fluorescence with different wavelength can be screened in a high-throughput manner.

Example VIII: Protein Array Using Spores Displaying on Its Surface Protein of Interest

106-109 spores displaying monoclonal antibodies against surface antigen of HBV are attached onto glass substrate for protein array (BMS, Germany) with aldehyde functional group on its surface using automated array apparatus. The attachment is made in a form of covalent linkage, which is Schiff base between amino group of protein on spore surface and aldehyde group on surface of slide glass. Although the displayed proteins attached on solid surface may be inactivated, they may have an orientation.

The protein array kit manufactured according to the present invention has a variety of applicable fields including diagnosis, analysis of gene expression, analysis of interaction between proteins, analysis of interaction

between protein and ligand, study on metabolism, screening novel or improved enzymes, combinatorial biochemical synthesis and biosensor.

Example IX: Production of Antibody Using Spores Displayig Antigen

The spores on its surface displaying surface antigen of HBV isolated in Example VII are suspended in PBS and the same volume of complete Freund's adjuvant is added. Thereafter, the mixture is well agitated to make emulsion formulation and the emulsion is injected i.v. into BALB/c mice with age of 6-8 week. After 4 weeks of the injection, the secondary administration is performed. Then, the additional boosting injection is performed about 2-3 times for induction of antibody.

As described above, the display method on spore surface of the present invention provides improvements in: a resistance against physiochemical change in environment of display host, a diversity of displayable proteins, a viability of display host and rapidity of screening.

Throughout this application, various patents and publications are referenced and citations are provided in parentheses. The disclosure of these patents and

publications in their entities are hereby incorporated by references into this application in order to more fully describe this invention and the state of the art to which this invention pertains.

Having described a preferred embodiment of the present invention, it is to be understood that variants and modifications thereof falling within the spirit of the invention may become apparent to those skilled in this art, and the scope of this invention is to be determined by appended claims and their equivalents.

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BUDAPEST TREATY ON THE INTERNATIONAL RECOGNITION OF THE DEPOSIT OF MICROORGANISMS FOR THE PURPOSE OF PATENT PROCEDURE

INTERNATIONAL FORM

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT

issued pursuant to Rule 7.1

TO : PAN, Jae-Gu
#380-43, Doryong-dong, Yusong-ku, Taejon 305-340,
Republic of Korea

I. IDENTIFICATION OF THE MICROORGANISM

Identification reference given by the DEPOSITOR:	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY:
Bacillus subtilis GFSD18	**KCTC 0887BP**

II. SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I above was accompanied by:
[x] a scientific description
[ ] a proposed taxonomic designation
(Mark with a cross where applicable)

III. RECEIPT AND ACCEPTANCE

This International Depositary Authority accepts the microorganism identified under I above, which was received by it on **November 13 2000.**

IV. RECEIPT OF REQUEST FOR CONVERSION

The microorganism identified under I above was received by this International Depositary Authority on and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name: Korean Collection for Type Cultures	Signature(s) of person(s) having the power to represent the International Depositary Authority of authorized official(s):
Address: Korea Research Institute of Bioscience and Biotechnology (KRIBB) #52, Oun-dong, Yusong-ku, Taejon 305-333, Republic of Korea	Bae K.S. BAE, Kyung Sook, Director Date: **November 16 2000**

Form BP/4 KCTC Form 17 sole page

What is claimed is:

1. A method for displaying a protein of interest on spore surface, which comprises the steps of:

(i) preparing a vector for spore surface display comprising a gene construct containing a gene encoding spore coat protein and a gene encoding a protein of interest, wherein, when expressed, the gene construct expresses a fusion protein between the spore coat protein and the protein of interest;

(ii) transforming a host cell with the vector for spore surface display;

(iii) displaying the protein of interest on a surface of a spore of the host cell; and

(iv) recovering the spore displaying on its surface the protein of interest.

2. A method for improving a protein of interest, which comprises the steps of:

(i) constructing a gene library of the protein of interest;

(ii) preparing a vector by linking the gene library to a gene encoding spore coat protein;

(iii) transforming a spore-forming host cell with the vector;

(iv) forming a spore in the transformed host cell and

displaying the protein of interest on a surface of the spore;

(v) recovering the spore displaying on its surface the protein of interest; and

(vi) screening the spore displaying a variant of the protein of interest having a desired property.

3. The method according to claim 2, wherein the screening is performed by means of measuring an activity of the protein or flow cytometry.

4. A method for improving a protein of interest using a resistance property of spore, which comprises the steps of:

(i) constructing a gene library of the protein of interest;

(ii) preparing a vector by linking the gene library to a gene encoding spore coat protein;

(iii) transforming a spore-forming host cell with the vector;

(iv) forming a spore in the transformed host cell and displaying the protein of interest on a surface of the spore;

(v) treating the spore displaying on its surface the protein of interest with one or more selected from the group consisting of organic solvent, heat, acid, base,

oxidant, dryness, surfactant and protease;

(vi) recovering the spore displaying on its surface the protein of interest; and

(vii) screening the spore displaying a variant of the protein of interest having a resistance to the treatment.

5. The method according to claim 4, wherein the screening is performed using an activity of the protein or a structural stability of the protein.

6. A method for bioconversion, which comprises the steps of:

(i) preparing a vector for spore surface display comprising a gene construct containing a gene encoding spore coat protein and a gene encoding a protein of interest conducting a bioconversion reaction, wherein, when expressed, the gene construct expresses a fusion protein between the spore coat protein and the protein of interest;

(ii) transforming a host cell with the vector for spore surface display;

(iii) displaying the protein of interest on a surface of a spore of the host cell;

(iv) recovering the spore displaying on its surface the protein of interest; and

(v) performing the bioconversion reaction using the spore

displaying on its surface the protein of interest.

7. A method for preparing protein microarray, which comprises the steps of:

(i) preparing a vector for spore surface display comprising a gene construct containing a gene encoding spore coat protein and a gene encoding antibody or antigen having binding affinity to a protein to be analyzed, wherein, when expressed, the gene construct expresses a fusion protein between the spore coat protein and the antibody or antigen;

(ii) transforming a host cell with the vector for spore surface display;

(iii) displaying the antibody or antigen on a surface of a spore of the host cell;

(iv) recovering the spore displaying on its surface the antibody or antigen; and

(v) immobilizing onto a solid surface the spore displaying on its surface the antibody or antigen.

8. A method producing an antibody to antigen in vertebrates, which comprises the steps of:

(i) preparing a vector for spore surface display comprising a gene construct containing a gene encoding spore coat protein and a gene encoding the antigen,

wherein, when expressed, the gene construct expresses a fusion protein between the spore coat protein and the antigen;

(ii) transforming a host cell with the vector for spore surface display;

(iii) displaying the antigen on a surface of a spore of the host cell;

(iv) recovering the spore displaying on its surface the antigen; and

(v) administering to vertebrates a composition containing an immunologically effective amount of the spore displaying on its surface the antigen.

9. A method for preparing a whole cell absorber, which comprises the steps of:

(i) preparing a vector for spore surface display comprising a gene construct containing a gene encoding spore coat protein and a gene encoding a protein having a binding affinity to a certain substance, wherein, when expressed, the gene construct expresses a fusion protein between the spore coat protein and the protein;

(ii) transforming a host cell with the vector for spore surface display;

(iii) displaying the protein on a surface of a spore of the host cell;

(iv) recovering the spore displaying on its surface the protein; and

(v) immobilizing onto a carrier the spore displaying on its surface the protein.

10. The method according to any one of claims 1-9, wherein the gene encoding spore coat protein is derived from a spore-forming Gram negative bacterium including *Myxococcus*, a spore-forming Gram positive bacterium including *Bacillus*, a spore-forming *Actionmycete*, a spore-forming yeast or a spore-forming fungus.

11. The method according to claim 10, wherein the gene encoding spore coat protein is derived from a spore-forming Gram positive bacterium.

12. The method according to claim 11, wherein the gene encoding spore coat protein is derived from *Bacillus*.

13. The method according to any one of claims 1-9, wherein the gene encoding spore coat protein is selected from the group consisting of cotA, cotB, cotC, cotD, cotE, cotF, cotG, cotH, cotJA, cotJC, cotK, cotL, cotM, cotS, cotT, cotV, cotW, cotX, cotY, cotZ, spoIVA, spoVID and sodA.

14. The method according to any one of claims 1-9, wherein the gene encoding spore coat protein is selected from the group consisting of cotA, cotE, cotF, cotG, cotH, cotJA, cotJC, cotK, cotL, cotM, cotS, cotT, cotV, cotW, cotX, cotY, cotZ, spoIVA, spoVID and sodA.

15. The method according to any one of claims 1-9, wherein the gene encoding spore coat protein is a modified form or a recombinant form of one selected from the group consisting of cotA, cotB, cotC, cotD, cotE, cotF, cotG, cotH, cotJA, cotJC, cotK, cotL, cotM, cotS, cotT, cotV, cotW, cotX, cotY, cotZ, spoIVA, spoVID and sodA, in which the modified form or the recombinant form has a more compatibility for spore surface display relative to wild type genes.

16. The method according to claim 15, wherein the modified form of the gene encoding spore coat protein is obtained by a method selected from the group consisting of DNA shuffling method, StEP method, RPR method, molecular breeding method, ITCHY method, error-prone PCR, point mutagenesis, nucleotide mutagenesis, combinatorial cassette mutagenesis and other suitable random mutagenesis.

17. The method according to any one of claims 1-9, wherein the gene encoding spore coat protein is selected from the

group consisting of cotA, cotB, cotC, cotD, cotE, cotF, cotG, cotH, cotJA, cotJC, cotK, cotL, cotM, cotS, cotT, cotV, cotW, cotX, cotY, cotZ, spoIVA, spoVID and sodA, in which the gene has a substituted promoter for its promoter to enhance spore surface display relative to wild type genes.

18. The method according to claim 13, wherein the gene encoding spore coat protein is cotE or cotG.

19. The method according to claim 14, wherein the gene encoding spore coat protein is cotE or cotG.

20. The method according to claim 15, wherein the gene encoding spore coat protein is cotE or cotG.

21. The method according to claim 16, wherein the gene encoding spore coat protein is cotE or cotG.

22. The method according to claim 16, wherein the gene encoding spore coat protein is cotE or cotG.

23. The method according to any one of claims 1-5, wherein the protein of interest is selected from the group consisting of enzyme, enzyme inhibitor, hormone, hormone analogue, hormone receptor, signal transduction protein,

antibody, monoclonal antibody, antigen, adhesive protein, structural protein, regulatory protein, toxin protein, cytokine, transcription regulatory protein, blood clotting protein, plant protection-inducing protein and fragments thereof.

24. The method according to any one of claims 1-9, wherein the host cell is selected from the group consisting of a spore-forming Gram negative bacterium including *Myxococcus*, a spore-forming Gram positive bacterium including *Bacillus*, a spore-forming *Actionmycete*, a spore-forming yeast or a spore-forming fungus.

25. The method according to claim 24, wherein the host cell is a spore-forming Gram positive bacterium.

26. The method according to claim 25, wherein the host cell is *Bacillus*.

27. The method according to any one of claims 1-9, wherein the spore is reproductive or non-reproductive one.

28. The method according to any one of claims 1-9, wherein the recovering is performed in such a manner that the display of the protein of interest on the spore surface is

maximized by regulating culture time, after which culturing is terminated and the spore is then recovered.

29. The method according to any one of claims 2-5, wherein the constructing a gene library is performed by a mutagenesis of the gene encoding the protein of interest of wild type, in which the mutagenesis is selected from the group consisting of DNA shuffling method, StEP method, RPR method, molecular breeding method, ITCHY method, error-prone PCR, point mutagenesis, nucleotide mutagenesis, combinatorial cassette mutagenesis and other suitable random mutagenesis.

30. A microbial transformant for spore surface display of a protein of interest, characterized in that the transformant is produced by transformation with a vector for spore surface display containing (i) a gene encoding a protein of interest and (ii) a gene encoding spore coat protein is selected from the group consisting of cotA, cotB, cotC, cotD, cotE, cotF, cotG, cotH, cotJA, cotJC, cotK, cotL, cotM, cotS, cotT, cotV, cotW, cotX, cotY, cotZ, spoIVA, spoVID and sodA, in which when expressed, a fusion protein between the spore coat protein and the protein of interest is expressed.

31. The transformant according to claim 30, wherein the

transformant is derived from a variant mutated to enhance spore surface display.

32. The transformant according to claim 31, wherein the mutation to enhance spore surface display eliminates a production of extracellular secretory protease in the transformant, so that the protein of interest displayed on spore surface is stably maintained.

33. The transformant according to claim 31, wherein the mutation to enhance spore surface display eliminates a production of intracellular protease in the transformant.

34. A spore for spore surface display of a protein of interest, characterized in that the spore displays the protein of interest on its surface.

35. The spore according to claim 34, wherein the spore is reproductive or non-reproductive one.

36. The spore according to claim 35, wherein the spore is non-reproductive one by virtue of one or more methods selected from the group consisting of genetic method, chemical method and physical method.

37. The spore according to claim 36, wherein the genetic method to make the spore non-reproductive is accomplished by deleting a gene involved in reproduction of spore.

38. The spore according to claim 34, wherein the spore is derived from a variant mutated to increase its agglutination property.

39. The spore according to claim 38, wherein the increase of the agglutination property in the spore is accomplished by one or more methods selected from the group consisting of genetic method, chemical method and physical method.

40. A vector for spore surface display, characterized in that the vector comprises a replication origin, an antibiotic-resistance gene, a restriction site, a gene encoding a spore coat protein, a gene encoding a protein of interest and a promoter operatively linked to the gene encoding a spore coat protein, in which when expressed, a fusion protein between the spore coat protein and the protein of interest is expressed.

41. The vector according to claim 40, wherein the gene encoding a spore coat protein is selected from the group consisting of cotA, cotB, cotC, cotD, cotE, cotF, cotG, cotH,

cotJA, cotJC, cotK, cotL, cotM, cotS, cotT, cotV, cotW, cotX, cotY, cotZ, spoIVA, spoVID and sodA.

42. The vector according to claim 41, wherein the gene encoding a spore coat protein is cotE or cotG.

FIG.1

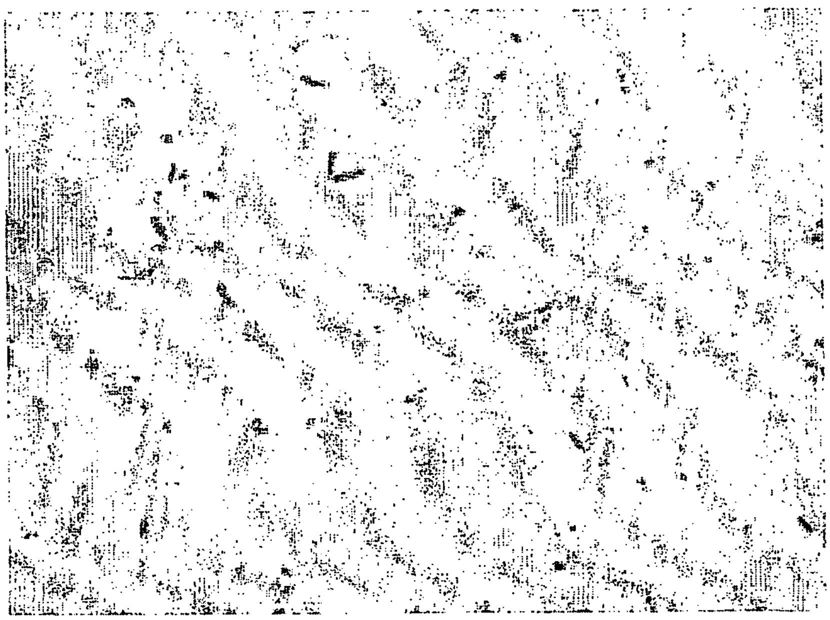


FIG.2

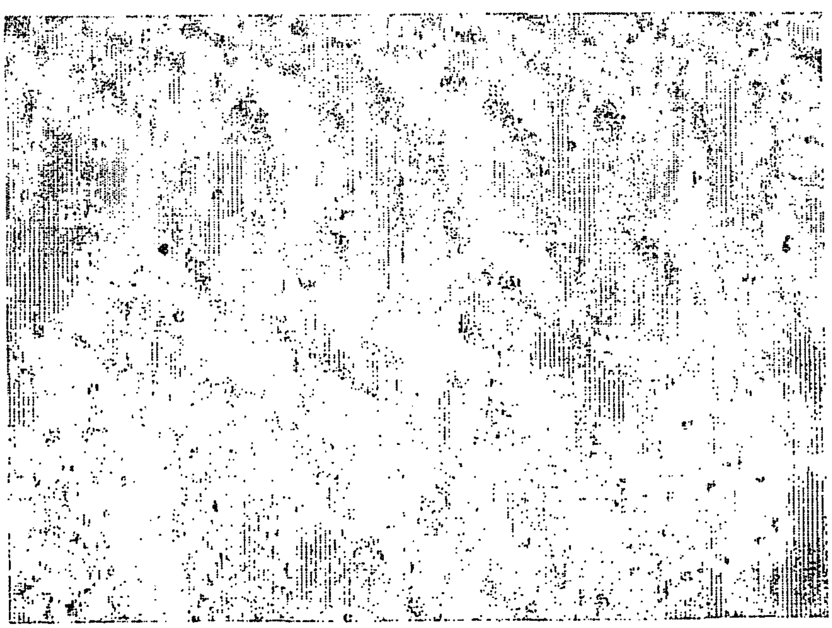


FIG.3

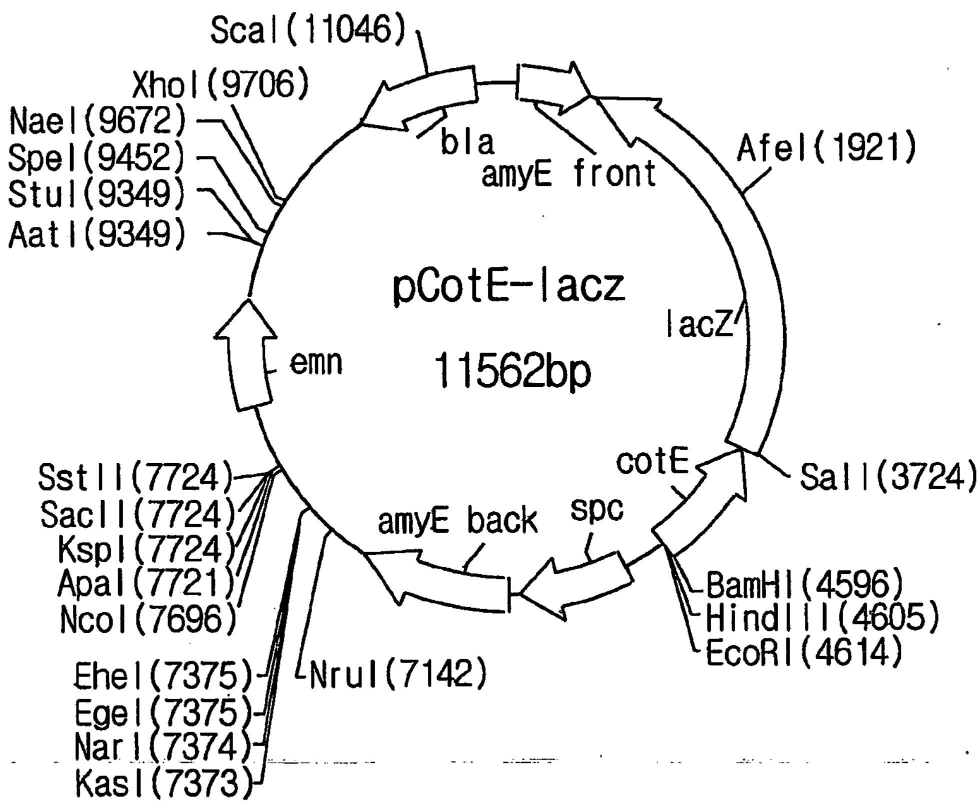

ScaI(11046)
XhoI(9706)
NaeI(9672)
SpeI(9452)
StuI(9349)
AatI(9349)
bla
amyE front
AfeI(1921)
pCotE-lacz
11562bp
lacZ
emn
cotE
SalI(3724)
spc
amyE back
SstII(7724)
SacII(7724)
KspI(7724)
ApaI(7721)
NcoI(7696)
BamHI(4596)
HindIII(4605)
EcoRI(4614)
EheI(7375)
EgeI(7375)
NarI(7374)
KasI(7373)
NruI(7142)

FIG.4

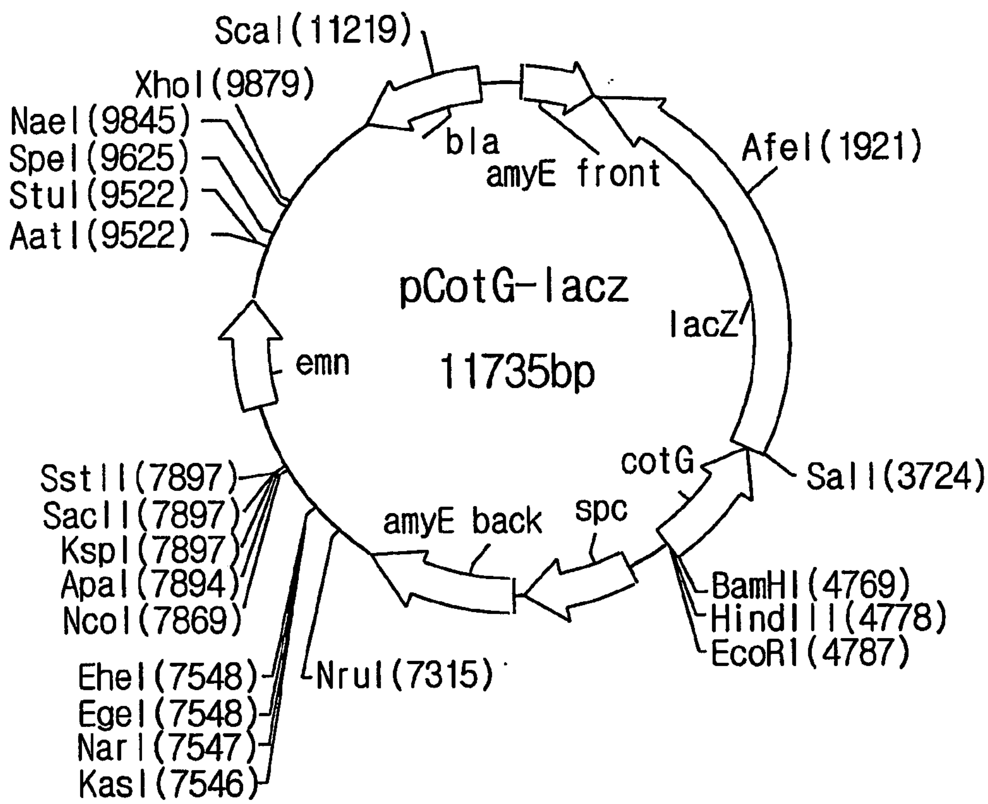

ScaI(11219)
XhoI(9879)
NaeI(9845)
SpeI(9625)
StuI(9522)
AatI(9522)
bla
amyE front
AfeI(1921)
pCotG-lacz
11735bp
lacZ
emn
cotG
SalI(3724)
spc
amyE back
SstII(7897)
SacII(7897)
KspI(7897)
ApaI(7894)
NcoI(7869)
BamHI(4769)
HindIII(4778)
EcoRI(4787)
EheI(7548)
EgeI(7548)
NarI(7547)
KasI(7546)
NruI(7315)

FIG.5

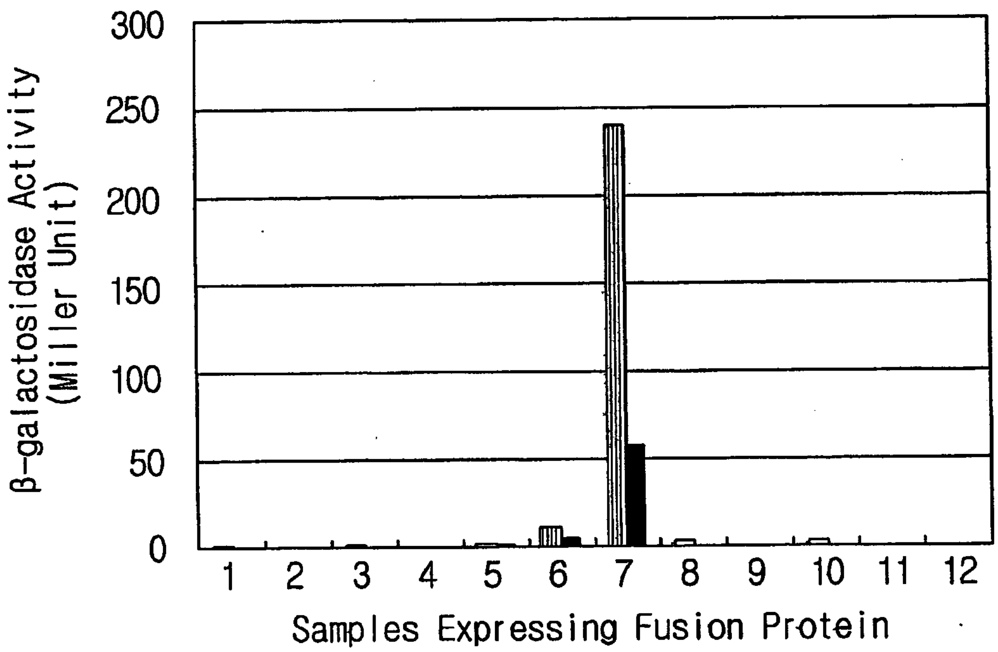

300
250
200
150
100
50
0
β-galactosidase Activity
(Miller Unit)
1 2 3 4 5 6 7 8 9 10 11 12
Samples Expressing Fusion Protein

FIG.6

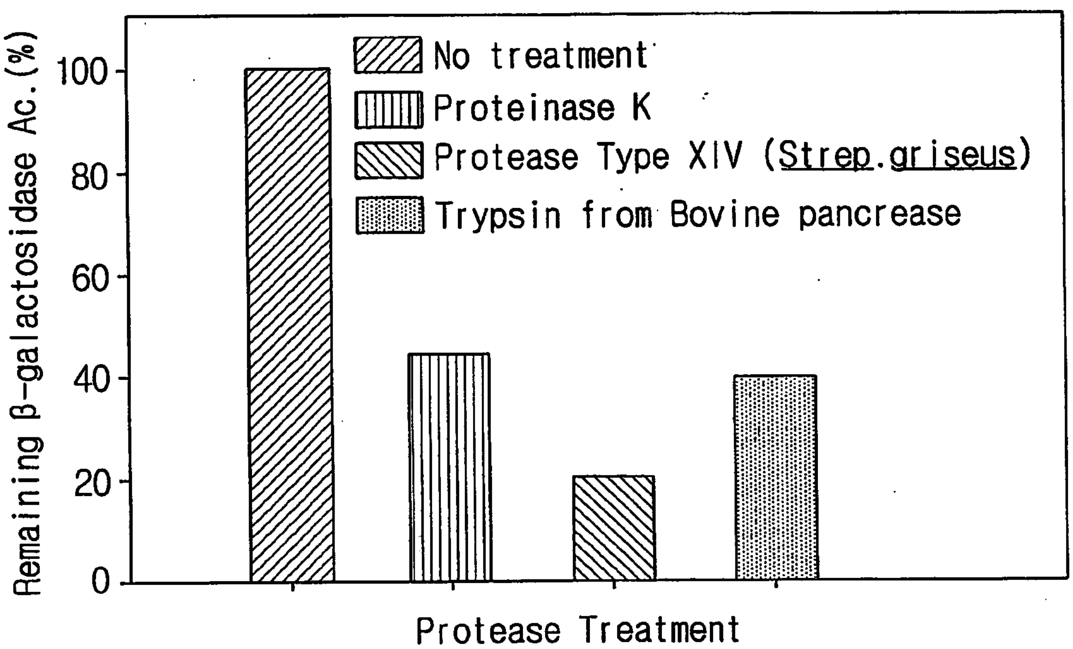
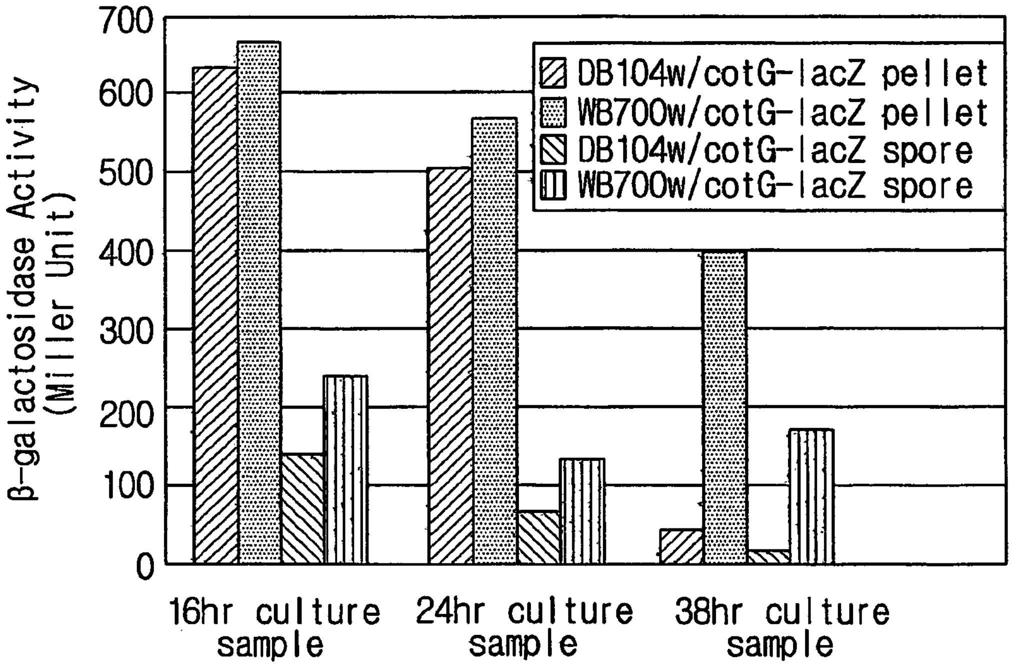

No treatment
Proteinase K
Protease Type XIV (Strep.griseus)
Trypsin from Bovine pancrease
Remaining β-galactosidase Ac.(%)
100
80
60
40
20
0
Protease Treatment

FIG.7



700
600
500
400
300
200
100
0
β-galactosidase Activity
(Miller Unit)
DB104w/cotG-lacZ pellet
WB700w/cotG-lacZ pellet
DB104w/cotG-lacZ spore
WB700w/cotG-lacZ spore
16hr culture sample
24hr culture sample
38hr culture sample

FIG.8

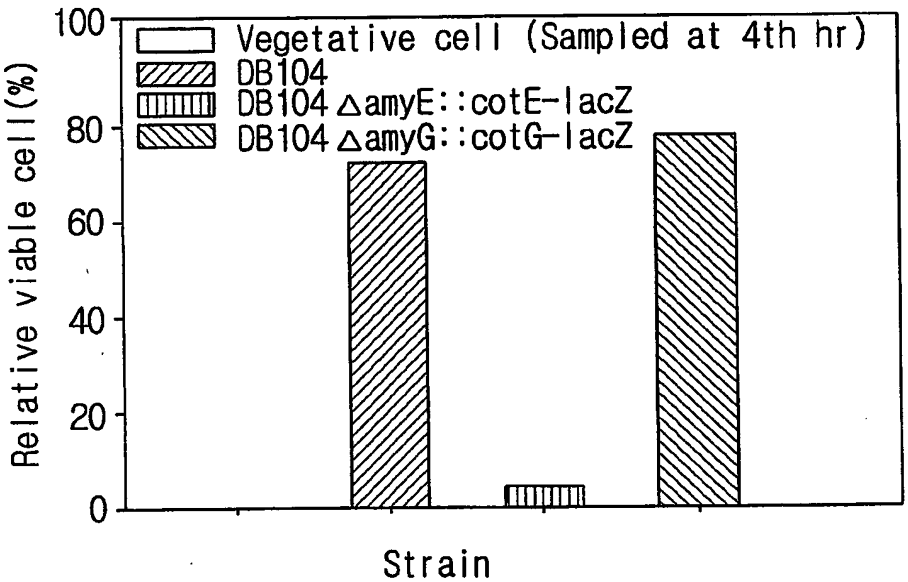

100
80
60
40
20
0
Relative viable cell(%)
Vegetative cell (Sampled at 4th hr)
DB104
DB104 △amyE::cotE-lacZ
DB104 △amyG::cotG-lacZ
Strain

FIG.9

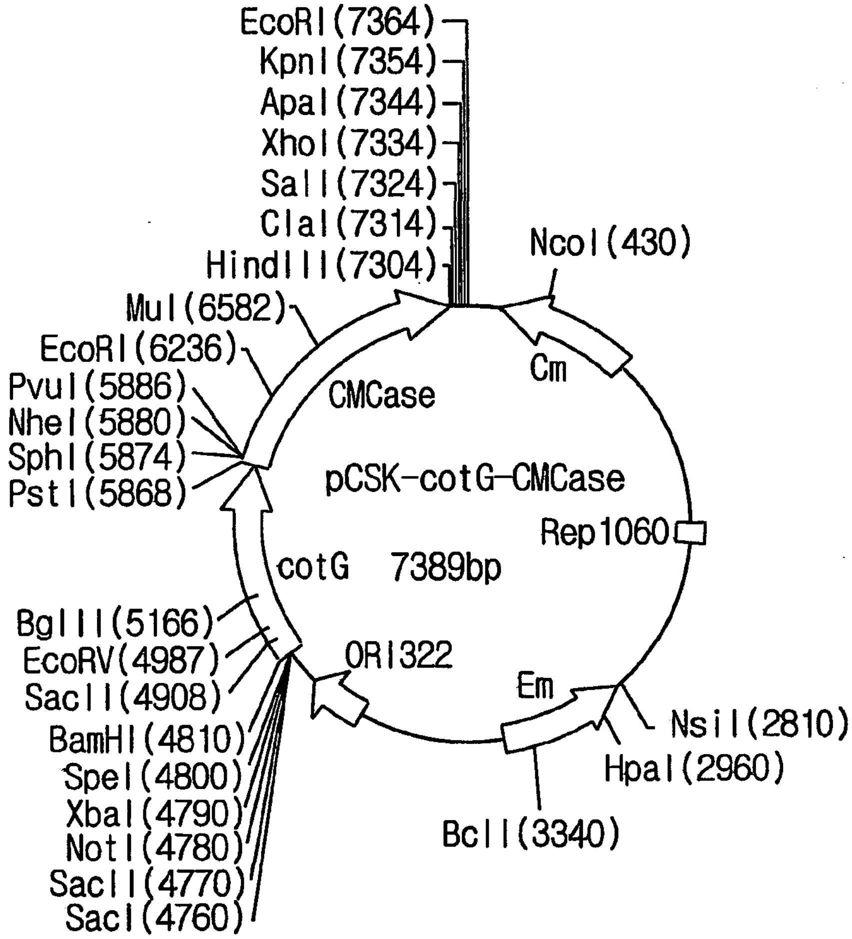

EcoRI(7364)
KpnI(7354)
ApaI(7344)
XhoI(7334)
SalI(7324)
ClaI(7314)
HindIII(7304)
NcoI(430)
MuI(6582)
EcoRI(6236)
PvuI(5886)
NheI(5880)
SphI(5874)
PstI(5868)
CMCase
Cm
pCSK-cotG-CMCase
Rep1060
cotG
7389bp
BglII(5166)
EcoRV(4987)
SacII(4908)
ORI322
Em
NsiI(2810)
HpaI(2960)
BamHI(4810)
SpeI(4800)
XbaI(4790)
NotI(4780)
SacII(4770)
SacI(4760)
BclI(3340)

FIG.10

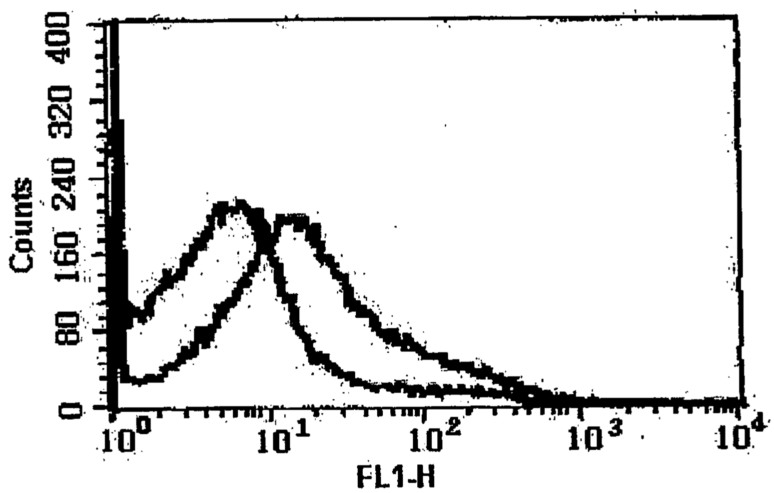

Counts
0
80
160
240
320
400
10^0
10^1
10^2
10^3
10^4
FL1-H

FIG.11

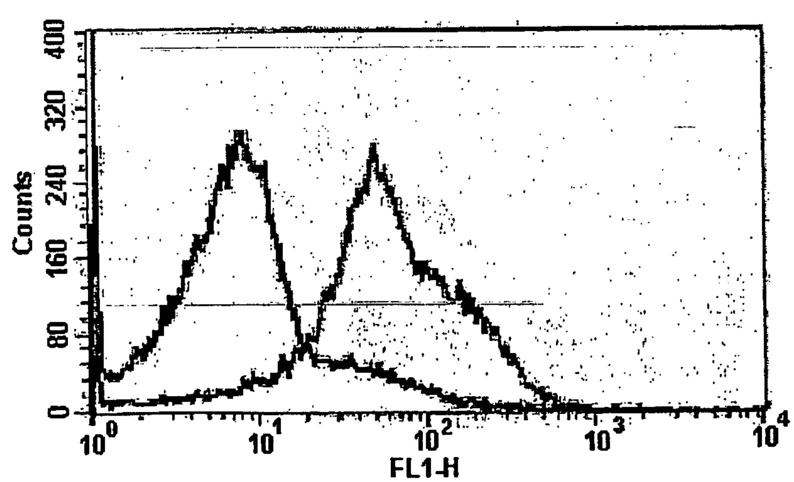

Counts
0
80
160
240
320
400
10^0
10^1
10^2
10^3
10^4
FL1-H

FIG.12

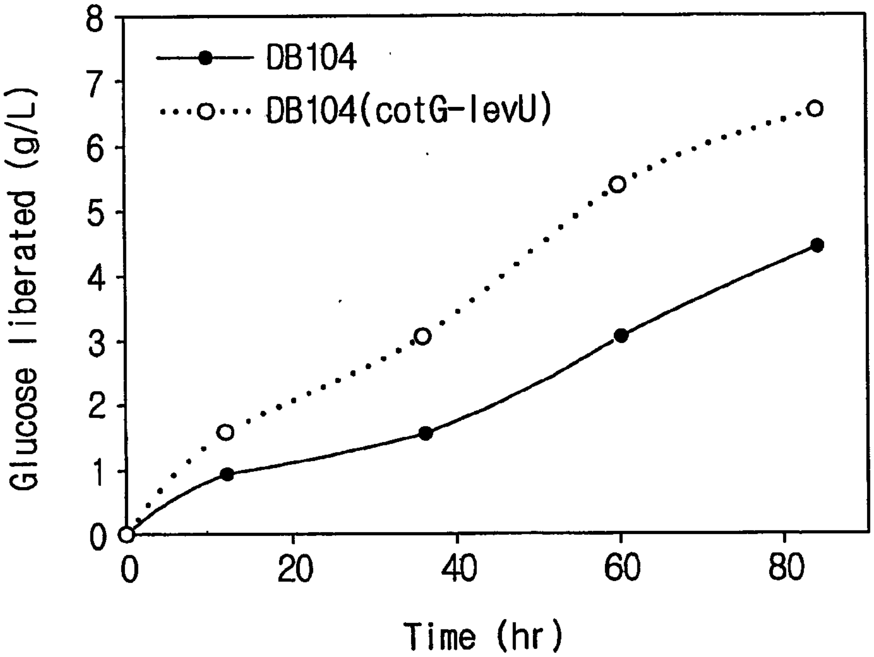

DB104
DB104(cotG-levU)
Glucose liberated (g/L)
Time (hr)
0
1
2
3
4
5
6
7
8
0
20
40
60
80

FIG.13

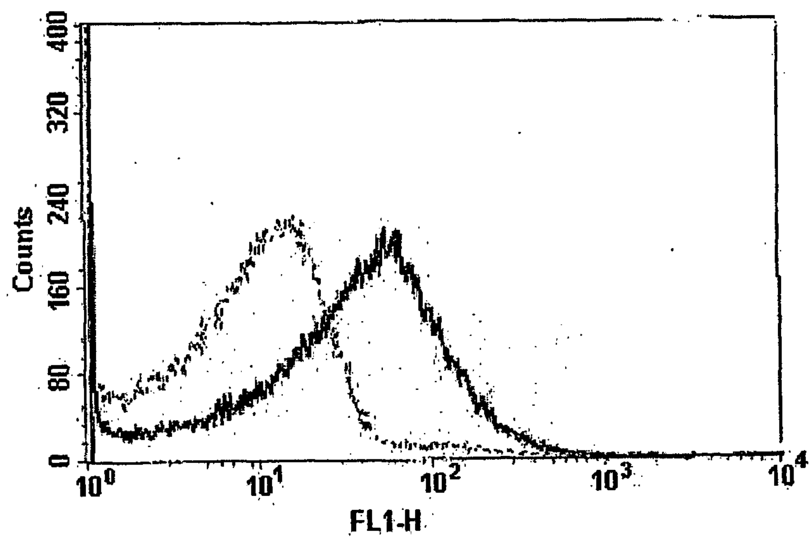


FIG.14

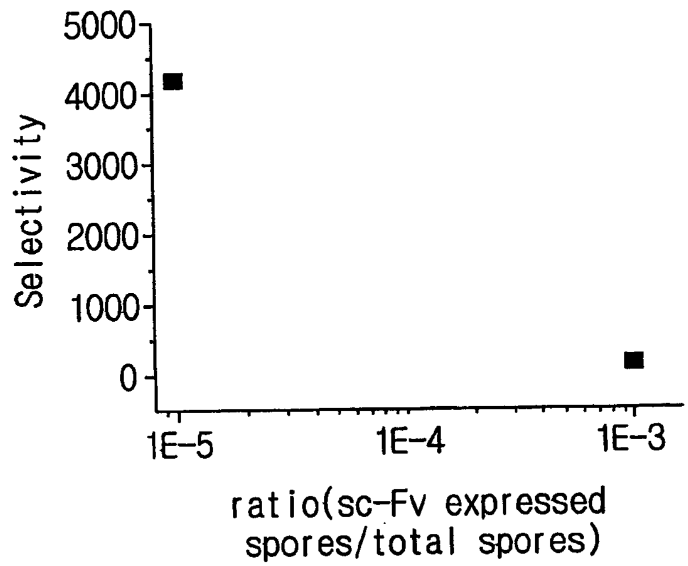

Selectivity
5000
4000
3000
2000
1000
0
1E-5
1E-4
1E-3
ratio(sc-Fv expressed spores/total spores)

FIG.15

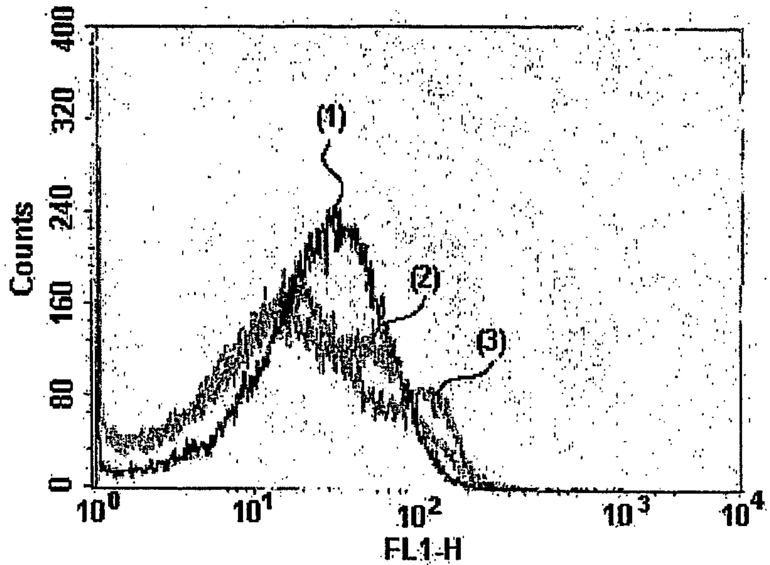

(1)
(2)
(3)
Counts
0
80
160
240
320
400
10^0
10^1
10^2
10^3
10^4
FL1-H

FIG.16

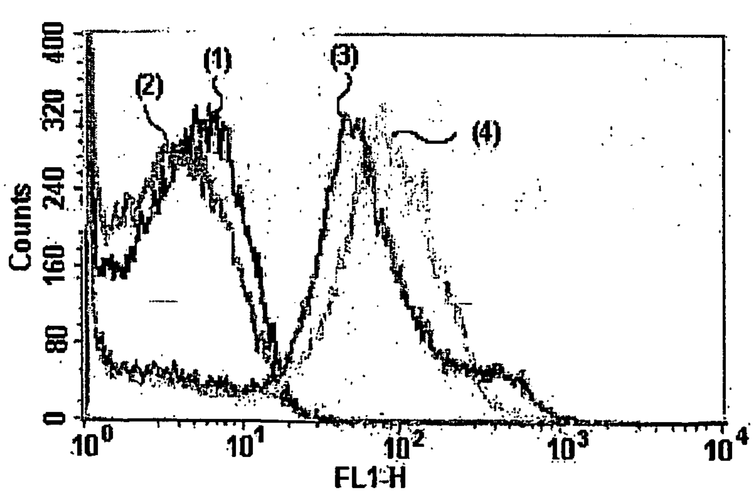

(2)
(1)
(3)
(4)
Counts
0
80
160
240
320
400
10^0
10^1
10^2
10^3
10^4
FL1-H

FIG.17a

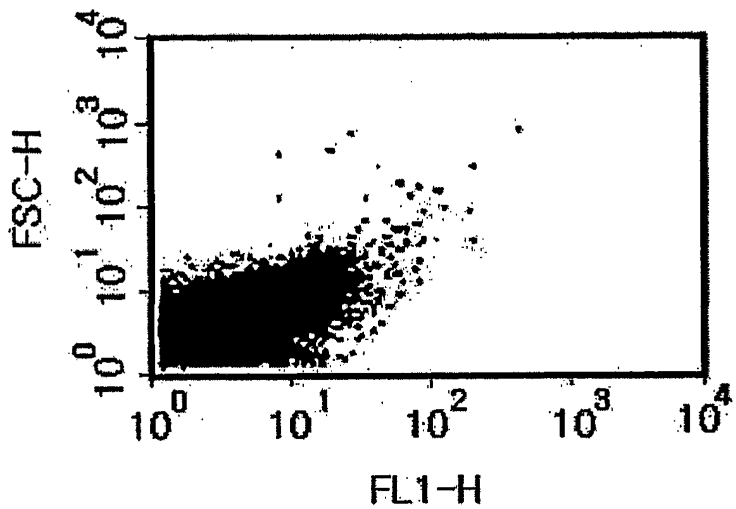


FIG.17b

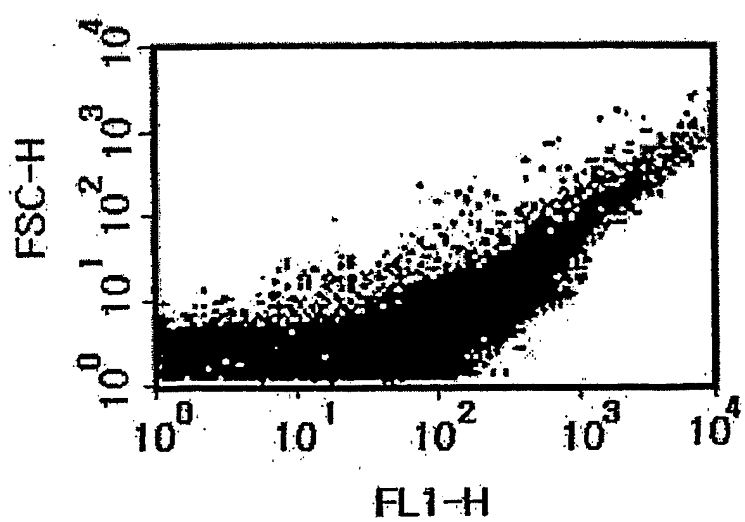


FIG.17c

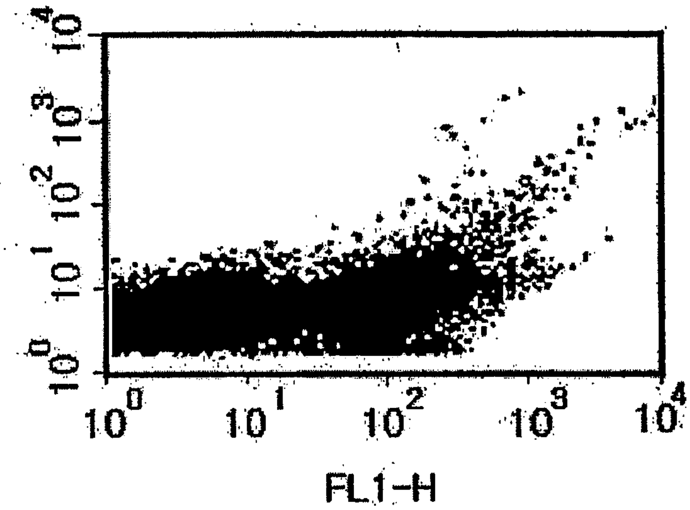
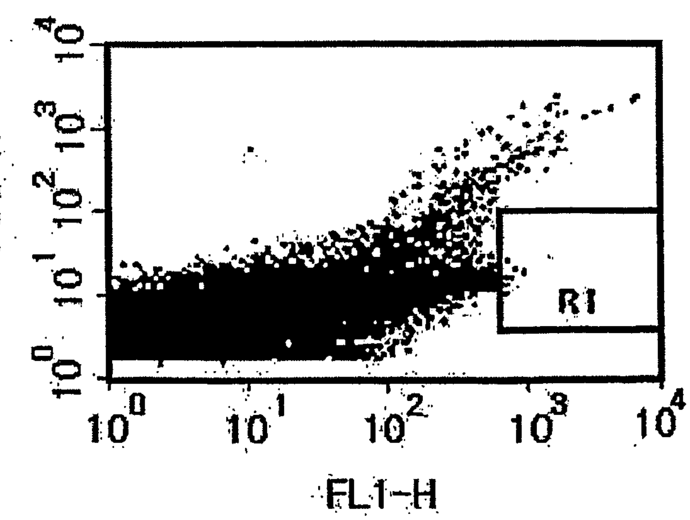


FIG.17d



<110> Genofocus Co., Ltd.

<120> Method for Expression of Proteins on Spore Surface

<130> PCT-Genofocus-1

<150> KR2000-74835
<151> 2000-12-08

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<170> KopatentIn 1.71

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<400> 2

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<220>
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<220>
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<210> 8
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<223> cotD 3' primer

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<212> DNA
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<220>
<223> cotH 5' primer

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<220>
<223> cotH 3' primer

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<210> 15
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<210> 16
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<212> DNA
<213> Artificial Sequence

<220>
<223> cotM 3' primer

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<210> 17
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<212> DNA
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<220>
<223> cotVWX 5' primer

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<210> 18
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<213> Artificial Sequence

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<210> 19
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<212> DNA
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<220>

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<400> 19

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<210> 20

<211> 36

<212> DNA

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<212> DNA
<213> Bacillus subtilis

<220>
<221> CDS
<222> (330)..(3902)

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ctctctaaac acggtgcctt tacaggcccg tgttttttta tcatttgtgc ggttaaaaat 120

gaactaaata atctatgtac caaatgttca attggttttt ctgtgctcag ccgcgtataa 180

actttatcgc acttataagt aaagtttcta ggcacccctg catacaatgg aacagaaact 240

ttgtattttt atattttatt tataaaaatg cacactagac aaatgcccag cataagataa 300

cacgaagaag aacaaggagg catgccgga atg tct gaa tac agg gaa att att 353
Met Ser Glu Tyr Arg Glu Ile Ile
1 5

acg aag gca gta gta gcg aaa ggc cga aaa ttc acc caa tgc acc aac 401

Thr Lys Ala Val Val Ala Lys Gly Arg Lys Phe Thr Gln Cys Thr Asn
10 15 20

acc atc tcg cct gag aaa aaa ccg agc agc att ttg ggt ggt tgg att 449
Thr Ile Ser Pro Glu Lys Lys Pro Ser Ser Ile Leu Gly Gly Trp Ile
25 30 35 40

att aac cac aag tat gac gct gaa aaa att gga aaa acg gta gaa att 497
Ile Asn His Lys Tyr Asp Ala Glu Lys Ile Gly Lys Thr Val Glu Ile
45 50 55

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Glu Gly Tyr Tyr Asp Ile Asn Val Trp Tyr Ser Tyr Ala Asp Asn Thr
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Lys Thr Glu Val Val Thr Glu Arg Val Lys Tyr Val Asp Val Ile Lys
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Leu Arg Tyr Arg Asp Asn Asn Tyr Leu Asp Asp Glu His Glu Val Ile
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Val Val Gly Glu Thr Lys Val Val Val Glu Val Asn Pro Asp Trp Glu
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Glu Asp Asp Glu Glu Asp Trp Glu Asp Glu Leu Asp Glu Glu Leu Glu
155 160 165

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Asp Ile Asn Pro Glu Phe Leu Val Gly Asp Pro Glu Glu Val Asp Arg
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Phe Ala Ser Trp Arg Asn Ser Glu Glu Ala Arg Thr Asp Arg Pro Ser
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Glu Ala Arg Thr Asp Arg Pro Ser Gln Gln Leu Arg Ser Leu Asn Gly
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Lys Pro Thr Thr Gln Ile Ser Asp Phe His Val Ala Thr Arg Phe Asn
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Glu Arg Gly Gly Tyr Ala Asp Arg Val Thr Leu Arg Leu Asn Val Glu
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Asn Pro Lys Leu Trp Ser Ala Glu Ile Pro Asn Leu Tyr Arg Ala Val
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Val Glu Leu His Thr Ala Asp Gly Thr Leu Ile Glu Ala Glu Ala Cys
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Asn Gly Lys Pro Leu Leu Ile Arg Gly Val Asn Arg His Glu His His
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His Ile Ser Ala Trp Gln Gln Trp Arg Leu Ala Glu Asn Leu Ser Val
885 890 895

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Leu Gly His Tyr Ser
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His Ser Asp Ile Glu Glu Ala Val Lys Ser Ala Lys Lys Glu Gly Leu
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Pro Phe Val Pro Thr Glu Asn Pro Thr Gly Cys Tyr Ser Leu Thr Phe
295 300 305

aat gtt gat gaa agc tgg cta cag gaa ggc cag acg cga att att ttt 1435
Asn Val Asp Glu Ser Trp Leu Gln Glu Gly Gln Thr Arg Ile Ile Phe
310 315 320 325

gat ggc gtt aac tcg gcg ttt cat ctg tgg tgc aac ggg cgc tgg gtc 1483
Asp Gly Val Asn Ser Ala Phe His Leu Trp Cys Asn Gly Arg Trp Val
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Gly Tyr Gly Gln Asp Ser Arg Leu Pro Ser Glu Phe Asp Leu Ser Ala
345 350 355

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Phe Leu Arg Ala Gly Glu Asn Arg Leu Ala Val Met Val Leu Arg Trp
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Ser Asp Gly Ser Tyr Leu Glu Asp Gln Asp Met Trp Arg Met Ser Gly
375 380 385

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Ile Phe Arg Asp Val Ser Leu Leu His Lys Pro Thr Thr Gln Ile Ser
390 395 400 405

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Asp Phe His Val Ala Thr Arg Phe Asn Asp Asp Phe Ser Arg Ala Val
410 415 420

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425 430 435

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Val Thr Val Ser Leu Trp Gln Gly Glu Thr Gln Val Ala Ser Gly Thr
440 445 450

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Ala Pro Phe Gly Gly Glu Ile Ile Asp Glu Arg Gly Gly Tyr Ala Asp
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Glu Ile Pro Asn Leu Tyr Arg Ala Val Val Glu Leu His Thr Ala Asp
490 495 500

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Gly Thr Leu Ile Glu Ala Glu Ala Cys Asp Val Gly Phe Arg Glu Val
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Arg Gly Val Asn Arg His Glu His His Pro Leu His Gly Gln Val Met
535 540 545

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Asp Glu Gln Thr Met Val Gln Asp Ile Leu Leu Met Lys Gln Asn Asn
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Thr Leu Cys Asp Arg Tyr Gly Leu Tyr Val Val Asp Glu Ala Asn Ile
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600 605 610

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Trp Leu Pro Ala Met Ser Glu Arg Val Thr Arg Met Val Gln Arg Asp
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His Gly Ala Asn His Asp Ala Leu Tyr Arg Trp Ile Lys Ser Val Asp
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Pro Ser Arg Pro Val Gln Tyr Glu Gly Gly Gly Ala Asp Thr Thr Ala
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Thr Asp Ile Ile Cys Pro Met Tyr Ala Arg Val Asp Glu Asp Gln Pro
680 685 690

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695 700 705

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730 735 740

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Pro Arg Leu Gln Gly Gly Phe Val Trp Asp Trp Val Asp Gln Ser Leu
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760 765 770

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Phe Gly Asp Thr Pro Asn Asp Arg Gln Phe Cys Met Asn Gly Leu Val
775 780 785

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810 815 820

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Ser Glu Tyr Leu Phe Arg His Ser Asp Asn Glu Leu Leu His Trp Met
825 830 835

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Val Ala Leu Asp Gly Lys Pro Leu Ala Ser Gly Glu Val Pro Leu Asp
840 845 850

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Pro Glu Ser Ala Gly Gln Leu Trp Leu Thr Val Arg Val Val Gln Pro
870 875 880 885

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Asn Ala Thr Ala Trp Ser Glu Ala Gly His Ile Ser Ala Trp Gln Gln
890 895 900

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gcc atc ccg cat ctg acc acc agc gaa atg gat ttt tgc atc gag ctg 3259
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Met Trp Ile Gly Asp Lys Lys Gln Leu Leu Thr Pro Leu Arg Asp Gln
950 955 960 965

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Phe Thr Arg Ala Pro Leu Asp Asn Asp Ile Gly Val Ser Glu Ala Thr
970 975 980

cgc att gac cct aac gcc tgg gtc gaa cgc tgg aag gcg gcg ggc cat 3451
Arg Ile Asp Pro Asn Ala Trp Val Glu Arg Trp Lys Ala Ala Gly His
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Tyr Gln Ala Glu Ala Ala Leu Leu Gln Cys Thr Ala Asp Thr Leu Ala
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Asp Ala Val Leu Ile Thr Thr Ala His Ala Trp Gln His Gln Gly Lys
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acc tta ttt atc agc cgg aaa acc tac cgg att gat ggt agt ggt caa 3595
Thr Leu Phe Ile Ser Arg Lys Thr Tyr Arg Ile Asp Gly Ser Gly Gln
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atg gcg att acc gtt gat gtt gaa gtg gcg agc gat aca ccg cat ccg 3643
Met Ala Ile Thr Val Asp Val Glu Val Ala Ser Asp Thr Pro His Pro
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Ala Arg Ile Gly Leu Asn Cys Gln Leu Ala Gln Val Ala Glu Arg Val
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Asn Trp Leu Gly Leu Gly Pro Gln Glu Asn Tyr Pro Asp Arg Leu Thr
1080 1085 1090

gcc gcc tgt ttt gac cgc tgg gat ctg cca ttg tca gac atg tat acc 3787
Ala Ala Cys Phe Asp Arg Trp Asp Leu Pro Leu Ser Asp Met Tyr Thr
1095 1100 1105

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Pro Tyr Val Phe Pro Ser Glu Asn Gly Leu Arg Cys Gly Thr Arg Glu
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Ser Arg Tyr Ser Gln Gln Gln Leu Met Glu Thr Ser His Arg His Leu
1145 1150 1155

ctg cac gcg gaa gaa ggc aca tgg ctg aat atc gac ggt ttc cat atg 3979
Leu His Ala Glu Glu Gly Thr Trp Leu Asn Ile Asp Gly Phe His Met
1160 1165 1170

ggg att ggt ggc gac gac tcc tgg agc ccg tca gta tcg gcg gaa ttt 4027
Gly Ile Gly Gly Asp Asp Ser Trp Ser Pro Ser Val Ser Ala Glu Phe
1175 1180 1185

cag ctg agc gcc ggt cgc tac cat tac cag ttg gtc tgg tgt caa aaa 4075
Gln Leu Ser Ala Gly Arg Tyr His Tyr Gln Leu Val Trp Cys Gln Lys
1190 1195 1200 1205

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35 40 45

His Lys Lys Ser Tyr Cys Ser His Lys Lys Ser Arg Ser His Lys Lys
50 55 60

Ser Phe Cys Ser His Lys Lys Ser Arg Ser His Lys Lys Ser Tyr Cys
65 70 75 80

Ser His Lys Lys Ser Arg Ser His Lys Lys Ser Tyr Arg Ser His Lys
85 90 95

Lys Ser Arg Ser Tyr Lys Lys Ser Tyr Arg Ser Tyr Lys Lys Ser Arg
100 105 110

Ser Tyr Lys Lys Ser Cys Arg Ser Tyr Lys Lys Ser Arg Ser Tyr Lys
115 120 125

Lys Ser Tyr Cys Ser His Lys Lys Lys Ser Arg Ser Tyr Lys Lys Ser
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Cys Arg Thr His Lys Lys Ser Tyr Arg Ser His Lys Lys Tyr Tyr Lys
145 150 155 160

Lys Pro His His His Cys Asp Asp Tyr Lys Arg His Asp Asp Tyr Asp
165 170 175

Ser Lys Lys Glu Tyr Trp Lys Asp Gly Asn Cys Trp Val Val Lys Lys
180 185 190

Lys Tyr Lys Val Asp Arg Glu Asn Pro Gly Val Thr Gln Leu Asn Arg
195 200 205

Leu Ala Ala His Pro Pro Phe Ala Ser Trp Arg Asn Ser Glu Glu Ala
210 215 220

Arg Thr Asp Arg Pro Ser Gln Gln Leu Arg Ser Leu Asn Gly Glu Trp
225 230 235 240

Arg Phe Ala Trp Phe Pro Ala Pro Glu Ala Val Pro Glu Ser Trp Leu
245 250 255

Glu Cys Asp Leu Pro Glu Ala Asp Thr Val Val Val Pro Ser Asn Trp
260 265 270

Gln Met His Gly Tyr Asp Ala Pro Ile Tyr Thr Asn Val Thr Tyr Pro
275 280 285

Ile Thr Val Asn Pro Pro Phe Val Pro Thr Glu Asn Pro Thr Gly Cys
290 295 300

Tyr Ser Leu Thr Phe Asn Val Asp Glu Ser Trp Leu Gln Glu Gly Gln
305 310 315 320

Thr Arg Ile Ile Phe Asp Gly Val Asn Ser Ala Phe His Leu Trp Cys
325 330 335

Asn Gly Arg Trp Val Gly Tyr Gly Gln Asp Ser Arg Leu Pro Ser Glu
340 345 350

Phe Asp Leu Ser Ala Phe Leu Arg Ala Gly Glu Asn Arg Leu Ala Val
355 360 365

Met Val Leu Arg Trp Ser Asp Gly Ser Tyr Leu Glu Asp Gln Asp Met
370 375 380

Trp Arg Met Ser Gly Ile Phe Arg Asp Val Ser Leu Leu His Lys Pro
385 390 395 400

Thr Thr Gln Ile Ser Asp Phe His Val Ala Thr Arg Phe Asn Asp Asp
405 410 415

Phe Ser Arg Ala Val Leu Glu Ala Glu Val Gln Met Cys Gly Glu Leu
420 425 430

Arg Asp Tyr Leu Arg Val Thr Val Ser Leu Trp Gln Gly Glu Thr Gln
435 440 445

Val Ala Ser Gly Thr Ala Pro Phe Gly Gly Glu Ile Ile Asp Glu Arg
450 455 460

Gly Gly Tyr Ala Asp Arg Val Thr Leu Arg Leu Asn Val Glu Asn Pro
465 470 475 480

Lys Leu Trp Ser Ala Glu Ile Pro Asn Leu Tyr Arg Ala Val Val Glu
485 490 495

Leu His Thr Ala Asp Gly Thr Leu Ile Glu Ala Glu Ala Cys Asp Val
500 505 510

Gly Phe Arg Glu Val Arg Ile Glu Asn Gly Leu Leu Leu Leu Asn Gly
515 520 525

Lys Pro Leu Leu Ile Arg Gly Val Asn Arg His Glu His His Pro Leu
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His Gly Gln Val Met Asp Glu Gln Thr Met Val Gln Asp Ile Leu Leu
545 550 555 560

Met Lys Gln Asn Asn Phe Asn Ala Val Arg Cys Ser His Tyr Pro Asn
565 570 575

His Pro Leu Trp Tyr Thr Leu Cys Asp Arg Tyr Gly Leu Tyr Val Val
580 585 590

Asp Glu Ala Asn Ile Glu Thr His Gly Met Val Pro Met Asn Arg Leu
595 600 605

Thr Asp Asp Pro Arg Trp Leu Pro Ala Met Ser Glu Arg Val Thr Arg
610 615 620

Met Val Gln Arg Asp Arg Asn His Pro Ser Val Ile Ile Trp Ser Leu
625 630 635 640

Gly Asn Glu Ser Gly His Gly Ala Asn His Asp Ala Leu Tyr Arg Trp
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Ile Lys Ser Val Asp Pro Ser Arg Pro Val Gln Tyr Glu Gly Gly Gly
660 665 670

Ala Asp Thr Thr Ala Thr Asp Ile Ile Cys Pro Met Tyr Ala Arg Val
675 680 685

Asp Glu Asp Gln Pro Phe Pro Ala Val Pro Lys Trp Ser Ile Lys Lys
690 695 700

Trp Leu Ser Leu Pro Gly Glu Thr Arg Pro Leu Ile Leu Cys Glu Tyr
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Ala His Ala Met Gly Asn Ser Leu Gly Gly Phe Ala Lys Tyr Trp Gln
725 730 735

Ala Phe Arg Gln Tyr Pro Arg Leu Gln Gly Gly Phe Val Trp Asp Trp
740 745 750

Val Asp Gln Ser Leu Ile Lys Tyr Asp Glu Asn Gly Asn Pro Trp Ser
755 760 765

Ala Tyr Gly Gly Asp Phe Gly Asp Thr Pro Asn Asp Arg Gln Phe Cys
770 775 780

Met Asn Gly Leu Val Phe Ala Asp Arg Thr Pro His Pro Ala Leu Thr
785 790 795 800

Glu Ala Lys His Gln Gln Gln Phe Phe Gln Phe Arg Leu Ser Gly Gln
805 810 815

Thr Ile Glu Val Thr Ser Glu Tyr Leu Phe Arg His Ser Asp Asn Glu
820 825 830

Leu Leu His Trp Met Val Ala Leu Asp Gly Lys Pro Leu Ala Ser Gly
835 840 845

Glu Val Pro Leu Asp Val Ala Pro Gln Gly Lys Gln Leu Ile Glu Leu
850 855 860

Pro Glu Leu Pro Gln Pro Glu Ser Ala Gly Gln Leu Trp Leu Thr Val
865 870 875 880

Arg Val Val Gln Pro Asn Ala Thr Ala Trp Ser Glu Ala Gly His Ile
885 890 895

Ser Ala Trp Gln Gln Trp Arg Leu Ala Glu Asn Leu Ser Val Thr Leu
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Pro Ala Ala Ser His Ala Ile Pro His Leu Thr Thr Ser Glu Met Asp
915 920 925

Phe Cys Ile Glu Leu Gly Asn Lys Arg Trp Gln Phe Asn Arg Gln Ser
930 935 940

Gly Phe Leu Ser Gln Met Trp Ile Gly Asp Lys Lys Gln Leu Leu Thr
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Pro Leu Arg Asp Gln Phe Thr Arg Ala Pro Leu Asp Asn Asp Ile Gly
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980 985 990

Lys Ala Ala Gly His Tyr Gln Ala Glu Ala Ala Leu Leu Gln Cys Thr
995 1000 1005

Ala Asp Thr Leu Ala Asp Ala Val Leu Ile Thr Thr Ala His Ala Trp
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Gln His Gln Gly Lys Thr Leu Phe Ile Ser Arg Lys Thr Tyr Arg Ile
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Asp Thr Pro His Pro Ala Arg Ile Gly Leu Asn Cys Gln Leu Ala Gln
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Val Ala Glu Arg Val Asn Trp Leu Gly Leu Gly Pro Gln Glu Asn Tyr
1075 1080 1085

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Ser Asp Met Tyr Thr Pro Tyr Val Phe Pro Ser Glu Asn Gly Leu Arg
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Cys Gly Thr Arg Glu Leu Asn Tyr Gly Pro His Gln Trp Arg Gly Asp
1125 1130 1135

Phe Gln Phe Asn Ile Ser Arg Tyr Ser Gln Gln Gln Leu Met Glu Thr
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Ser His Arg His Leu Leu His Ala Glu Glu Gly Thr Trp Leu Asn Ile
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Asp Gly Phe His Met Gly Ile Gly Gly Asp Asp Ser Trp Ser Pro Ser
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Val Trp Cys Gln Lys
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taaaaggaaa tatggtatga cttcttttg aagtctctga tatgtgatcc ccgataagcg 180

atatcaatat ccagcctttt ttgatttacc ttcatcacag ctggcaccgg atcatcgtcc 240

catatatcct tttttaattc acgcaagtct tttggatgaa caaacagctg ataaagcggt 300

aaattggatt gattcttcat ccataatcct ccttacaaat tttaggcttt tatttttata 360

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atcttcatcc ttaacatatt tttaaaagga ggatttcaaa ttg ggc cac tat tcc 475
Leu Gly His Tyr Ser
1 5

cat tct gac atc gaa gaa gcg gtg aaa tcc gca aaa aaa gaa ggt tta 523
His Ser Asp Ile Glu Glu Ala Val Lys Ser Ala Lys Lys Glu Gly Leu
10 15 20

aag gat tat tta tac caa gag cct cat gga aaa aaa cgc agt cat aaa 571

Lys Asp Tyr Leu Tyr Gln Glu Pro His Gly Lys Lys Arg Ser His Lys
25 30 35

aag tcg cac cgc act cac aaa aaa tct cgc agc cat aaa aaa tca tac 619
Lys Ser His Arg Thr His Lys Lys Ser Arg Ser His Lys Lys Ser Tyr
40 45 50

tgc tct cac aaa aaa tct cgc agt cac aaa aaa tca ttc tgt tct cac 667
Cys Ser His Lys Lys Ser Arg Ser His Lys Lys Ser Phe Cys Ser His
55 60 65

aaa aaa tct cgc agc cac aaa aaa tca tac tgc tct cac aag aaa tct 715
Lys Lys Ser Arg Ser His Lys Lys Ser Tyr Cys Ser His Lys Lys Ser
70 75 80 85

cgc agc cac aaa aaa tcg tac cgt tct cac aaa aaa tct cgc agc tat 763
Arg Ser His Lys Lys Ser Tyr Arg Ser His Lys Lys Ser Arg Ser Tyr
90 95 100

aaa aaa tct tac cgt tct tac aaa aaa tct cgt agc tat aaa aaa tct 811
Lys Lys Ser Tyr Arg Ser Tyr Lys Lys Ser Arg Ser Tyr Lys Lys Ser
105 110 115

tgc cgt tct tac aaa aaa tct cgc agc tac aaa aag tct tac tgt tct 859
Cys Arg Ser Tyr Lys Lys Ser Arg Ser Tyr Lys Lys Ser Tyr Cys Ser
120 125 130

cac aag aaa aaa tct cgc agc tat aag aag tca tgc cgc aca cac aaa 907
His Lys Lys Lys Ser Arg Ser Tyr Lys Lys Ser Cys Arg Thr His Lys
135 140 145

aaa tct tat cgt tcc cat aag aaa tac tac aaa aaa ccg cac cac cac 955
Lys Ser Tyr Arg Ser His Lys Lys Tyr Tyr Lys Lys Pro His His His
150 155 160 165

tgc gac gac tac aaa aga cac gat gat tat gac agc aaa aaa gaa tac 1003

Cys Asp Asp Tyr Lys Arg His Asp Asp Tyr Asp Ser Lys Lys Glu Tyr
170 175 180

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Trp Lys Asp Gly Asn Cys Trp Val Val Lys Lys Lys Tyr Lys Gly Gly
185 190 195

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Gly Gly Ser Leu Gln Ala Cys Ala Ser Arg Ser Gly Thr Lys Thr Pro
200 205 210

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Val Ala Lys Asn Gly Gln Leu Ser Ile Lys Gly Thr Gln Leu Val Asn
215 220 225

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Arg Asp Gly Lys Ala Val Gln Leu Lys Gly Ile Ser Ser His Gly Leu
230 235 240 245

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Gln Trp Tyr Gly Glu Tyr Val Asn Lys Asp Ser Leu Lys Trp Leu Arg
250 255 260

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Asp Asp Trp Gly Ile Thr Val Phe Arg Ala Ala Met Tyr Thr Ala Asp
265 270 275

ggc ggt ata att gac aac ccg tcc gtg aaa aat aaa atg aaa gaa gcg 1339
Gly Gly Ile Ile Asp Asn Pro Ser Val Lys Asn Lys Met Lys Glu Ala
280 285 290

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295 300 305

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Ile Leu Asn Asp Gly Asn Pro Asn Gln Asn Lys Glu Lys Ala Lys Glu
310 315 320 325

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Phe Phe Lys Glu Met Ser Ser Leu Tyr Gly Asn Thr Pro Asn Val Ile
330 335 340

tat gaa att gca aac gaa cca aac ggt gat gtg aac tgg aag cgt gat 1531
Tyr Glu Ile Ala Asn Glu Pro Asn Gly Asp Val Asn Trp Lys Arg Asp
345 350 355

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Ile Lys Pro Tyr Ala Glu Glu Val Ile Ser Val Ile Arg Lys Asn Asp
360 365 370

cca gac aac att atc att gtc gga acc ggt aca tgg agc cag gat gtg 1627
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR01/02124

A. CLASSIFICATION OF SUBJECT MATTER

IPC7 C12N 15/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(7) C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Korean patent and application for invention since 1975

Electronic data base consulted during the intertnational search (name of data base and, where practicable, search terms used)
Kipass, Medline, Delphion, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,766,914(Michigan State University), 16 Jun 1998 (see the whole document)	1-4,6,10-14,17-26 28, 30-34, 40-42
Y	Hiroshi Ichikawa et al., "Combined action of transcription factors regulates genes encoding spore coat proteins of Bacillus subtilis", J Bioll Chem., May 2000 275(18):13849-55 (see the whole document)	1-4,6,10-14,17-26 28, 30-34, 40-42
A	US 5,837,500(Dyax, Corp.) 17 Nov. 1998 (see the whole document)	7

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevence
"E" earlier application or patent but published on or after the international filing date
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"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevence; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevence; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents,such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search
30 APRIL 2002 (30.04.2002)

Date of mailing of the international search report
30 APRIL 2002 (30.04.2002)

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Facsimile No. 82-42-472-7140

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Telephone No. 82-42-481-5593

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/KR01/02124

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5,766,914	16.06.1998	WO 9623063 A1 EP 084784 A1	01.08.1996 13.05.1998
US 5,837,500	17,11,1998	WO 9002809 A1 EP 0436597 A1	16.04.1990 17.07.1991